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Docket No.: 204372000301
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Lynn E. SPITLER et al.

Application No.: 09/300,978

Filed: April 28, 1999

For: PROSTATIC CANCER VACCINE

Art Unit: 1644

Examiner: P. Gambel

REQUEST FOR REHEARING PURSUANT TO 37 C.F.R. § 1.197(b)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Decision on Appeal dated December 20, 2004, Applicants respectfully request that the above-identified Patent application be reconsidered in view of the remarks which follow, that each of the presently pending claims be allowed, and that the application be passed to issue. It is believed that this request is timely filed as it is filed within two months of the mailing date of the board's decision.

Also included herewith are the following Exhibits:

Exhibit A: *Immunity to Tumors* IN CELLULAR AND MOLECULAR IMMUNOLOGY 336 (Abbas et al., eds., W.B. Saunders Co. 1991).

Exhibit B: Kearny, *Idiotypic Networks* IN FUNDAMENTAL IMMUNOLOGY 887 (Paul, W.E., ed., Raven Press Ltd. 1993).

Exhibit C: Berzofsky et al., *Immunogenicity and Antigen Structure* IN FUNDAMENTAL IMMUNOLOGY 235 (Paul, W.E., ed., Raven Press Ltd. 1993).

Exhibit D: *Antigen Presentation and T Cell Antigen Recognition* IN CELLULAR AND MOLECULAR IMMUNOLOGY 116-17 (Abbas et al., eds., W.B. Saunders Co. 1991).

sd-239067

REMARKS

In accordance with 37 C.F.R. § 1.197(b), Applicants hereby request a rehearing of the decision mailed December 22, 2004 in the instant application with regard to pending claims 13, 15, 16, and 18-24. In this request, Applicants address below the points believed to be misapprended or overlooked in rendering the decision. Specifically, Applicants address:

- The definition of tumor associated antigens ("TAAs") has been misapprended and is taken out of context.
- The decision fails to appreciate that the immunogenicity of anti-idiotypic antibodies cannot predict the immunogenicity of a corresponding antigen in another species.

PSMA is not a tumor associated antigen as disclosed by Spitler

Applicants respectfully submit that the broad nature of the definition used by the Examiner and the Board misconstrues, and in fact contravenes, the meaning of TAAs thought to be useful in a vaccine by a person of skill in the art at the time of filing. Antigen expression and antigen immunogenicity in a vaccine are not synonymous. Even if the genus of TAAs as allegedly disclosed by Spitler included PSMA (whether it does or not), the success of this particular species is surprising and in contrast to the TAAs previously disclosed in the art because PSMA is normally overexpressed in non-cancer (or normal) tissue. This is surprising and unexpected as a person of skill in the art believed that antigens expressed in normal cells are not immunogenic. For example, one immunology textbook states:

> Tumors that arise from a certain tissue often express the differentiation antigens of that tissue. Since these antigens are part of normal cells, they do **not** stimulate immune responses against the tumors on which they are expressed.

See Exhibit A at 345 (emphasis added). Notably, the author lists PSA and PAP, as exemplary tissue-specific antigens that do **not** elicit anti-tumor responses in Table 17-2. *Id.* Thus, the person of skill in the art would not reasonably expect an antigen such as PSMA to be a tumor associated antigen useful in a vaccine without *some* specific teaching that this distinct class of antigens is included in the definition of tumor associated antigens.

The definition in Paul[1] the decision relied upon fails to provide any specific teaching that indicates that normally (or constitutively) expressed antigens from normal tissues are a suitable antigen in a tumor vaccine. In fact, Paul's definition lacks any suggestion tissue specific antigens are targets for immunotherapy. Paul discusses the potential for embryonic, fetal, and clonal antigens as immunotherapeutic targets, but the only use Paul discusses for tissue-specific antigens is for diagnostic purposes. This alone would not convey to the person of ordinary skill in the art that antigens normally expressed on non-cancer tissues are suitable for use in a tumor vaccine.

Furthermore, the use of the broad definition relied upon in the decision renders Spitler's vaccine potentially fatal and deeply flawed scientifically. To employ this broad definition, one assumes that Spitler teaches the use of any tissue specific antigen expressed by a tumor *regardless* of the outcome of any immune response. The claimed invention lies in recognition that normally expressed prostate specific antigens can result in the elimination of tumor through active immunotherapy in a manner that does not jeopardize the life of the patient. The ability to use such antigens is not taught or suggested in any combination of the references at issue.

Anti-idiotypic antibodies do not predict immune responsiveness to PSMA

Respectfully, the decision oversimplifies and misunderstands the requirements for an antigen expressed on normal tissue to elicit an effective antitumor response. While anti-idiotypic antibodies may be able to elicit an immune response, without more any immune response elicited by an anti-idiotypic antibody does not predict that PSMA alone is immunogenic or can elicit an effective antitumor response. This is because of fundamental differences in the initiation of the immune response to PSMA.

As a preliminary matter, Applicants believe an explanation of the anti-idiotypic antibody relied upon provides a context for the discussion which follows. An anti-idiotypic antibody (often referred to as the "Ab2" antibody) is specific for the idiotypic antibody (often referred to as the "Ab1" antibody). The Ab1 antibody is the antibody which originally binds the antigen. An anti-idiotypic antibody can be specific for any amino acid sequence (linear or conformational) of the Ab1 antibody. In some, but not all, instances the anti-idiotypic antibody specifically (and precisely) binds the antigen binding cleft of Ab1. When that happens, the antigen binding cleft of Ab2 should

[1] "Differentiation antigens and other tumor-associated antigens" IN FUNDAMENTAL IMMUNOLOGY 931-32 (Paul, W.E.,

be complementary to the antigen-binding cleft of Ab1 and theoretically be identical to epitope recognized by Ab1. However, because the antigen-binding cleft is *one of many* amino acid sequences presented by the Ab1 antibody, the anti-idiotypic antibody frequently to bind sites other than the antigen-binding cleft. *See* Exhibit B for a review.

Despite the logic of the idiotypic network described above when considered in isolation, the reality is necessarily more nuanced, less straightforward, and lacking the certainty assumed by the decision - a common situation for biology-based inventions. In order for an anti-idiotypic antibody to accurately predict immunogenicity and thereby substitute for the original antigen, it must have an amino acid sequence with the following characteristics:

- it is a sequence from the antigen;
- it does not contain foreign amino acid sequences; and
- it specifically elicits a T cell-mediated antitumor response.

It is highly unlikely that the amino acid sequence of the anti-idiotypic antibody is a sequence from the antigen. The anti-idiotypic antibody can bind **any** sequence in the Ab1 antibody and is more likely than not to bind sequences other than the antigen-binding cleft because the antigen-binding cleft represents a very small percentage of the overall antibody sequence. Therefore, the anti-idiotypic antibody that binds the antigen-binding cleft of the Ab1 perfectly **may** be present, but not easily identified without significant experimentation.

If the anti-idiotypic antibody is present and is identified, it may contain foreign amino acid sequences. The anti-idiotypic antibody is typically generated in a non-human species, *e.g.*, a mouse, using a non-human Ab1 antibody. There is **no** guarantee that the amino acid sequences have sufficient similarity to adequately mimic the antigen to elicit an antigen-specific antitumor response.

Assuming the epitope presented by the anti-idiotypic antibody has surmounted each of the hurdles discussed above, a epitope capable of eliciting an antibody (or B cell) response in both species does **not** predict that the same epitope will elicit a T cell response. At the time of filing, antibody responses to tumor antigens alone were not believed to be sufficient for tumor eradication. *See, e.g.*, Exhibit A at 347 (stating "[no] evidence exists, however, for a role of such humoral

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ed. Raven Press, NY 1989).

[antibody] responses in inhibiting tumor development or growth").[2] Therefore, the desired epitope was one recognized and responded to by T cells. B cells and T cells do not necessarily see the same epitopes. For example, in the textbook FUNDAMENTAL IMMUNOLOGY, it states:

> [in] contrast to T cell recognition of "processed" fragments retaining only primary and secondary structures, the evidence is overwhelming that most antibodies are made against the native conformation when the native protein is used as an immunogen.

See Exhibit C at 243 (emphasis added).[3] Because of the broader class of epitopes immunogenic to B cells, the ability to elicit an antibody to an epitope lacks any predictive value regarding the immunogenicity of such an antigen to T cells. As noted in one textbook:

> ...we do not yet know all the constraints on the relationships between T and B cell epitopes **and therefore cannot use this information in a predictive way**...

See Exhibit C at 274 (emphasis added).

Finally, the antigen offered by the anti-idiotypic antibody may not be the one offered by the antigen itself. In other words, even if the anti-idiotypic antibody is presumed to have sufficient amino acid sequence to mimic an amino acid sequence in the antigen, it is **not** predictable that this particular epitope is one that is immunogenic in a host of another species, *i.e.*, can effectively elicit an antitumor response in a patient. Because of differences in the antigen presentation molecules and processing between species, the ability of one species to select and present a particular amino acid sequence does not provide any predictive indication of the effectiveness of the same amino acid sequence to be selected and presented in another species. Thus, a highly immunogenic epitope in one species may elicit no response at all in another species.

At least some degree of predictability is required if the teachings of cited combination of references are to render the claimed methods obvious. At the time of filing, the numerous hurdles requiring investigation and experimentation prevented a person of ordinary skill in the art from extrapolating from anti-idiotypic antibodies to antigen-based active immunotherapy with any

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[2] Applicants note that the ability of an administered antibody to act as an effective tumor agent was not known until several years after the original 1993 filing date. The success of Her2 occurred after the 1993 filing date and was both unexpected and surprising.

[3] *See also* Exhibit D at 116 (stating that "T lymphocytes recognize different forms of antigens from B lymphocytes and secreted immunoglobulins").

certainty, therefore the combination of references fails to render the claimed methods *prima facie* obvious.

Accordingly, Applicants respectfully request a reconsideration of the board's decision regarding the obviousness rejection of the pending claims.

CONCLUSION

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 204372000301. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 22, 2005

Respectfully submitted,

By _____

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- [ ] Information Disclosure Statement
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- [x] Other Enclosure(s) (please Identify below):

Request for Rehearing Pursuant to 37 C.F.R. § 1.197(b) (7 pgs)
Exhibit A: Immunity to Tumors IN CELLULAR AND MOLECULAR IMMUNOLOGY 336 (Abbas et al., eds., W.B. Saunders Co. 1991) (20 pgs)
Exhibit B: Kearny, Idiotypic Networks IN FUNDAMENTAL IMMUNOLOGY 887 (Paul, W.E., ed., Raven Press Ltd. 1993) (18 pgs)
Exhibit C: Berzofsky et al., Immunogenicity and Antigen Structure IN FUNDAMENTAL IMMUNOLOGY 235 (Paul, W.E., ed., Raven Press Ltd. 1993) (48 pgs)
Exhibit D: Antigen Presentation and T Cell Antigen Recognition IN CELLULAR AND MOLECULAR IMMUNOLOGY 116-17 (Abbas et al., eds., W.B. Saunders Co. 1991) (2 pgs)
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Dated: February 22, 2005 Signature: _____ (Judy Bridgwater)

sd-245603

CELLULAR AND MOLECULAR IMMUNOLOGY

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CHAPTER SEVENTEEN

IMMUNITY TO TUMORS

Malignant tumors, or cancers, grow in an uncontrolled manner, invade normal tissues, and often metastasize and grow at sites distant from the tissue of origin. In general, cancers are derived from one or only a few normal cells that have undergone a poorly understood process called malignant transformation. Cancers can arise from almost any tissue in the body. Those derived from epithelial cells, called carcinomas, are the most common kinds of cancers. Sarcomas are malignant tumors of mesenchymal tissues, arising from cells such as fibroblasts, muscle cells, and fat cells. Solid malignant tumors of lymphoid tissues are called lymphomas, and marrow and blood-borne malignant tumors of lymphocytes and other hematopoietic cells are called leukemias.

Hypothetically, a major function of the immune system could be to recognize and destroy spontaneously arising malignantly transformed cells, so-called "mutant clones," before they grow into tumors. This idea, called **immunosurveillance,** was articulated by Macfarlane Burnet and Lewis Thomas in the 1950s and 1960s. In addition, immune responses to malignant cells may be protective even after these cells have grown into tumors. If malignant cells and tumors can stimulate immune responses, it follows that they must express **tumor antigens** that are recognized as foreign by the tumor-bearing host. Furthermore, if the concept of immunosurveillance is valid, immune effector cells, such as B cells, helper T cells, cytolytic T lymphocytes (CTLs), or natural killer (NK) cells must be able to recognize tumor antigens and mediate the killing of tumor cells.

A common histologic observation which suggests that tumors may be immunogenic is the presence of mononuclear cell infiltrates, composed of T cells, NK cells, and macrophages, surrounding many tumors. Although such infiltrates may often result after tissue destruction caused by the tumor, they are more frequently present around certain types of tumors, including testicular seminomas, thymomas, medullary breast carcinoma, and malignant melanomas in the skin, irrespective of the presence of other inflammatory stimuli such as infection or tissue necrosis. In fact, the presence of lymphocytic infiltrates in medullary breast carcinomas and malignant melanomas is associated with a better prognosis compared with histologically similar tumors without infiltrates. Another histopathologic indication that tumors stimulate immune responses is the frequent finding of lymphocytic proliferation (hyperplasia) in lymph node draining sites of tumor growth. Furthermore, there is often evidence of cytokine effects in tumors, such as class II MHC expression on tumor cells and endothelial cells of tumor vessels, suggesting an active immune response at the sites of the tumors.

At one time, the major effector mechanism for tumor immunosurveillance was considered to be the CTL. In fact, when CTLs were first discovered, their only demonstrated function was the artificial role of killing allogeneic cells in a transplant or a mixed leukocyte reaction (MLR), and antitumor activity was the assumed physiologic role for these cells. More recently, we have come to appreciate that CTLs are most important in anti-viral immunity. Furthermore, a critical evaluation of the immunosurveillance hypothesis suggests that it is not generally valid for most forms of cancer. For example, if the immune system is required to prevent the frequent occurrence of cancers, one would expect that many more malignant tumors would develop in individuals with congenital or acquired immunodeficiencies than in immunocompetent individuals. In fact, this is not the case for most common forms of cancers, such as carcinomas of the colon, lung, or breast. There is, however, a strikingly increased incidence of certain forms of cancer in immunosuppressed individuals, and many of these cancers may result from infections with tumor-causing viruses (as discussed later in the chapter). Thus, the concept of immunosurveillance may be most relevant for the limited subset of cancers caused by oncogenic viruses.

The idea that the immune system responds to tumors has served as the stimulus for a branch of immunology called **tumor immunology.** The field of tumor immunology encompasses the study of specific acquired immune responses to tumors, the antigens on tumor cells which induce immune responses, immunologic effector mechanisms that kill tumor cells, and immunologic approaches for detecting, diagnosing and treating cancers. The great progress we have made over the last decade in understanding the physiology of normal immune responses is already being applied to the important practical problems of prevention and treatment of tumors. This chapter discusses these different aspects of tumor immunology, referring to the basic principles of the cognitive and effector arms of the immune response which have already been described in detail in previous chapters.

TUMOR ANTIGENS

The abnormal growth behaviors of malignant tumors are the reflection of complex abnormalities in physiology which result from expression of mutated or viral genes and/or deregulated expression of normal genes. It is a reasonable assumption, therefore, that cancer cells express certain proteins that are either not expressed at all or are present in much lower quantities in normal cells. Such proteins may be the tumor antigens, that are seen as foreign by a tumor host, resulting in specific immune responses to tumor cells. In addition, surface proteins peculiar to tumors may serve as targets for effectors of natural immunity, such as NK cells.

The fact that tumor cells express antigens that can stimulate immune responses in the host has been clearly demonstrated in both experimental animal models and in human cancer patients. Two main approaches have been used to identify tumor antigens. First, antibodies can be produced by immunizing an animal with the tumor cells, and these antibodies can then be used as probes for different molecules expressed on the tumor cell surface. Second, tumor anti-

gens can be operationally defined as molecules that stimulate T cell-mediated rejection of tumor transplants in an animal previously immunized with the tumor. The tumor antigens that are defined by rejection experiments are called **tumor-specific transplantation antigens** (TSTAs); they include tumor cell proteins that have been processed and presented by the tumor cell in association with major histocompatibility complex (MHC) molecules. Some tumor antigens are unique to particular tumors, whereas others are present on many or all cancers of a specific type.

A fundamental, and as yet unanswered, challenge in the field of tumor immunology is to determine the role that immune responses to tumor antigens play in the control of naturally occurring tumors. For example, there is no compelling evidence that the presence of anti-tumor antibodies serves to block tumor growth. It is also clear that immune responses to TSTAs often do not block the outgrowth of artificially induced tumors and may be effective only in the experimental situation of tumor transplantation into a specifically immunized animal. It should be kept in mind, however, that our difficulty in demonstrating the physiologic significance of tumor antigens may be a reflection of our limited (but improving) ability to analyze the complex biology of tumor growth and *in vivo* immune responses.

This portion of the chapter describes several types of tumor antigens that have been studied in some detail, providing various insights into the biology of tumor-host interactions.

Unique Tumor Antigens

Studies in the 1950s of chemical-induced or radiation-induced tumors in inbred strains of mice demonstrated the existence of antigens expressed exclusively by the cells of one individual tumor. Although analogous unique tumor antigens have not been demonstrated in naturally occurring human tumors, this model of tumor antigens is significant because it is the clearest demonstration that the immune system can specifically prevent the growth of malignant tumors. In a typical study of this sort, a sarcoma is induced in an inbred mouse by painting its skin with the chemical carcinogen, methylcholanthrene (MCA). These MCA-induced sarcomas can be excised from the original host mouse and introduced into other mice or back into the original animal. Upon transplantation of these tumors into other syngeneic mice, the tumors grow and eventually kill the new host. In contrast, reintroduction of the tumor into the original host results in a specific immunologic rejection of the tumor. Adoptive transfer experiments show that rejection is mediated mainly by tumor-specific CTLs. Alternatively, the cells of a tumor from one mouse can be killed by irradiation and used to immunize a second syngeneic mouse. Subsequent introduction of live cells from the original tumor into the immunized mouse results in immunologic rejection of the tumor transplant (Fig. 17-1). These experiments demonstrate that the *rejection of the transplanted tumors displays two cardinal features of specific immune responses, namely specificity and memory.* In addition, they suggest that CTLs are an important effector mechanism for anti-tumor immunity. Since the tumor antigens in this experimental system are defined on the basis of rejection of transplanted tumor cells, they are called TSTAs. A remarkable property of these TSTAs is their enormous diversity, reflected by the specificity of the immune responses to each individual tumor. For example, one MCA-induced sarcoma does not induce protective immunity against another MCA-induced sarcoma, even if both tumors are derived from the same mouse (Table 17-1, experiment 1).

Although TSTAs were described over 30 years ago, attempts to define their molecular nature were for a long time largely unsuccessful. For many years, investigators tried, and failed, to raise monoclonal antibodies specific for these antigens. Recently, however, genes encoding some of these TSTAs have been identified. The strategy used was to artificially mutagenize a tumorigenic (tum^+) mouse cell line and isolate non-tumorigenic (tum^-) variant cell lines. It was established that the tum^- phenotype was due to the

TABLE 17-1. Transplantation Antigens on Chemically and Virally Induced Tumors

Experiment	Treatment of Mouse: *Immunization with Killed Tumor Cells from*	Treatment of Mouse: *Challenge with Live Tumor Cells from*	Result	Conclusion
1	Chemically induced sarcoma A	Chemically induced sarcoma A	No growth	Immunity to chemically induced tumors is specific for individual tumors.
	Chemically induced sarcoma A	Chemically induced sarcoma B	Growth of chemically induced sarcoma B	
2	MSV-induced sarcoma A	MSV-induced sarcoma A	No growth	Immunity to virus-induced tumors is virus-specific.
	MSV-induced sarcoma A	MSV-induced sarcoma B	No growth of sarcoma B	
	MSV-induced sarcoma A	Chemically induced sarcoma C	Growth of chemically induced sarcoma C	
	MSV-induced sarcoma A	MuLV-induced sarcoma D	Growth of MuLV-induced sarcoma D	

Abbreviations: MSV, murine sarcoma virus; MuLV, murine leukemia virus.

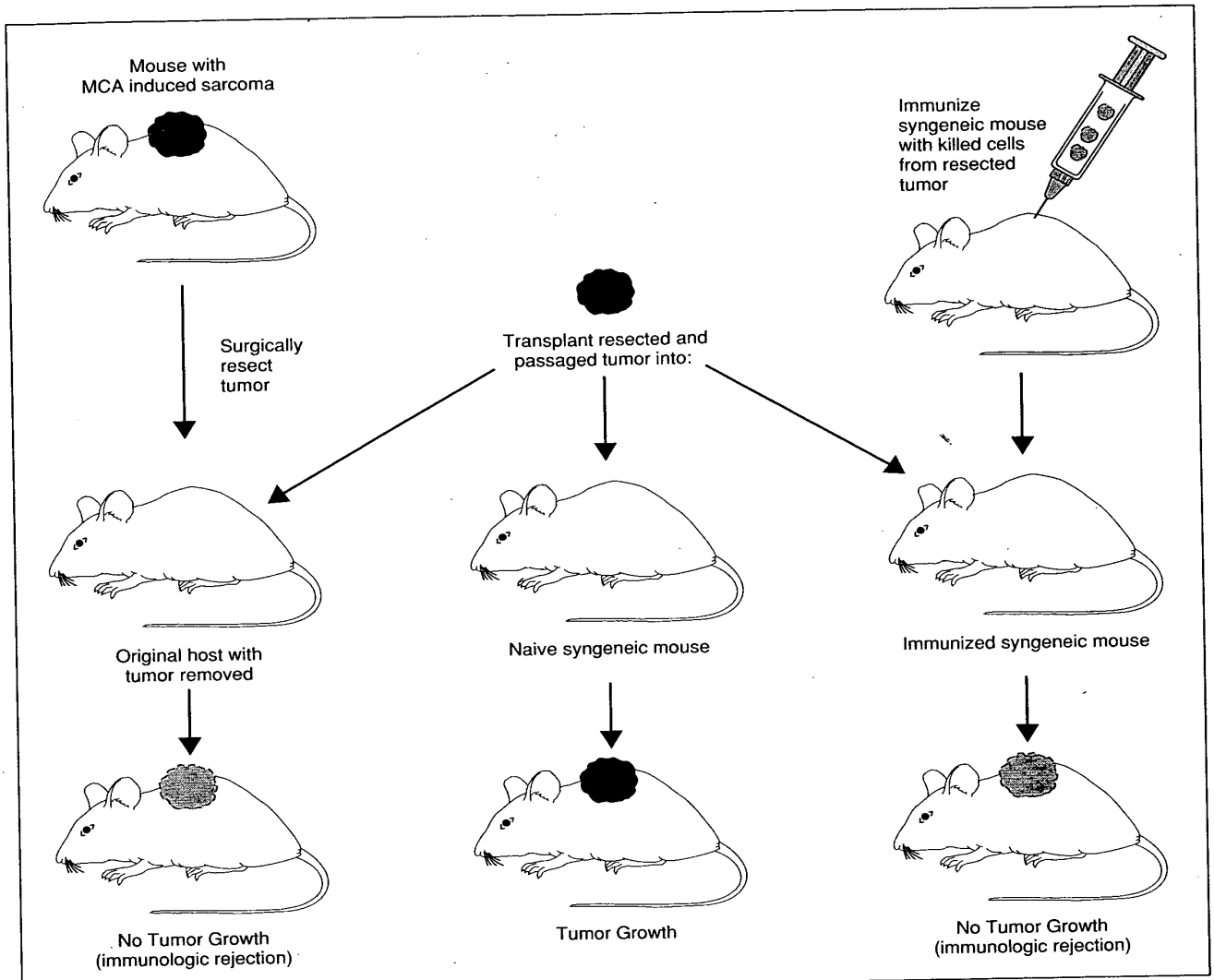

FIGURE 17–1. Tumor-specific transplantation antigens (TSTAs) of chemically induced sarcomas. *A mouse treated with the chemical carcinogen methylcholanthrene (MCA) develops a sarcoma. If this tumor is resected and transplanted into a normal syngeneic mouse, the tumor will grow. In contrast, the original tumor-bearing animal that was cured by surgical resection will reject a subsequent transplant of the same tumor. Injection of killed cells from the same tumor into a syngeneic mouse induces the same type of protective immunity.*

presence of unique TSTAs that were not present on the parent tum^+ cells. These TSTAs stimulated a specific CTL-mediated rejection of transplanted tum^- cells. In other words, the tum^- variant expresses TSTAs and is immunogenic whereas the tum^+, TSTA-negative parent line is not immunogenic. A cosmid library of genes was derived from the tum^- line, and these genes were then transfected into the tum^+ line. By this approach, genes were identified that encoded the TSTAs and that would convert the cells to a nontumorigenic, i.e., immunogenic, phenotype (Fig. 17–2). In this system, the TSTA-encoding genes are highly diverse and apparently represent point mutations of various unrelated normal cellular genes. It is plausible that a similarly diverse group of mutated genes code for the unique TSTAs in the MCA-sarcoma model described above.

The proteins produced by such tumor-specific genes are endogenously synthesized, processed, and presented to the host immune system, usually in association with class I MHC molecules. The processing and presentation of endogenously synthesized proteins presumably occurs normally in many or all cells. If the proteins are normal self proteins, they do not induce immune responses because of the absence of self reactive T cells. If, however, the proteins are altered or mutated forms of normal proteins, they will be recognized by specific CTLs and will serve as targets for cell lysis and rejection. Thus, *in different tumors, the TSTAs may be altered forms of different cellu-*

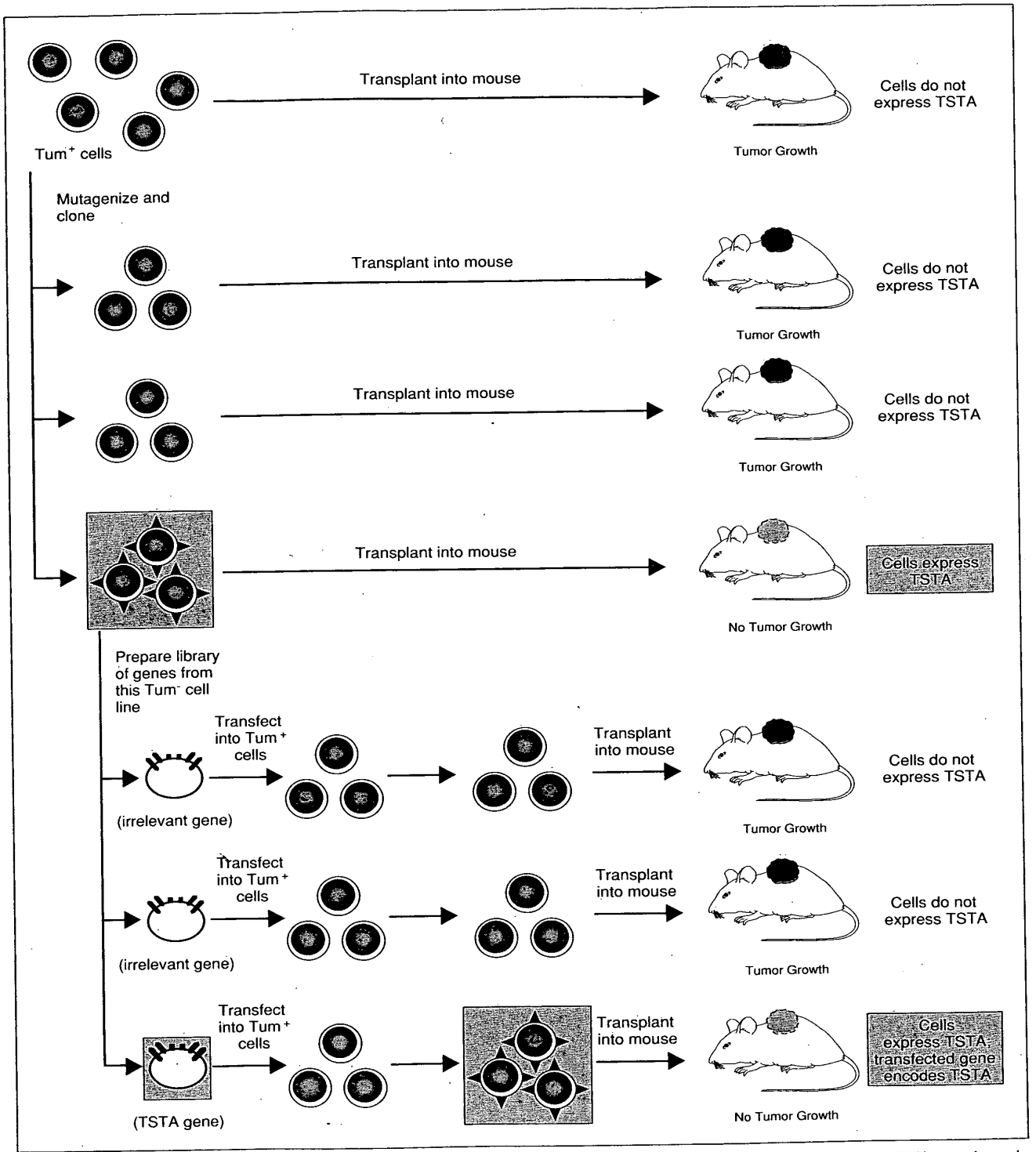

FIGURE 17-2. Identification of genes encoding tumor-specific transplantation antigens (TSTAs). *Cells expressing TSTAs and genes encoding TSTAs are shown in shaded boxes. The genes identified by this approach encode a variety of apparently unrelated cellular proteins, with point mutations resulting in one or a few amino acid differences from normal proteins. These mutated proteins induce immune responses that result in tumor transplant rejection.*

lar proteins. The fact that most of these unique TSTAs are only present on the cell surface in the form of peptides bound to MHC molecules is probably why it has been difficult to raise antibodies against them. The relationship (if any) of the mutations to the malignant phenotype of the tumor cells is not known. The mutations may be a result of the high doses of mutagens or carcinogens used to generate the experimental tumors and tumor variants. A similar rate of mutations and the resultant expression of unique tumor antigens may not occur in human tumors, since human tumors are rarely caused by such high doses of mutagens.

Antigens Shared by Different Tumors

Most tumor antigens studied are not unique to individual tumors, but are shared by different tumors. Furthermore, most, if not all, of these diverse groups of antigens may be found on normal cells or benign tumor cells. Such antigens are often called **tumor-associated antigens** (TAAs). There are several classes of these antigens, and many different ones may be expressed on the same tumor. An example of the diversity of TAAs is seen in the phenotype of human melanomas (Box 17-1).

Silent Genes

Some cellular genes that are not normally expressed in an individual may become transcriptionally active in tumor cells, and therefore their protein products may be recognized by the host as foreign antigens. These genes are called "silent" genes. For example, the non-polymorphic class I MHC-like molecule called the thymic leukemia antigen (Tla) is not normally expressed at any time in some strains of mice, whereas other strains express the antigen on developing thymocytes. T cell leukemias from any strain of mice invariably express Tla molecules, and these antigens can elicit antibody responses in normally Tla-negative mice. Tla, however, does not appear to elicit tumor rejection responses, and its role in protective immunity is not known. Human counterparts of tumor-associated silent gene products have not yet been described.

Oncofetal Antigens

Oncofetal antigens make up another class of tumor-associated antigens. They are normally ex-

BOX 17-1. MELANOMA ANTIGENS

Malignant melanomas are aggressive, frequently metastatic and fatal tumors derived from either melanocytes or melanocyte related nevus cells. They make up 3 per cent of all skin cancers. Melanomas are among the most thoroughly characterized human tumors with respect to surface antigen expression. This is a reflection of the facts that the primary skin tumors are frequently excised and that melanoma cells can be grown in tissue culture more easily than many other tumor types. Furthermore, melanoma cells express several highly immunogenic surface molecules, including chondroitin sulfate proteoglycan and gangliosides. The fact that melanomas can stimulate immune responses *in vivo* is suggested by the frequent presence of intense lymphocytic infiltrates adjacent to these tumors.

The major strategy for characterizing melanoma antigens is to immunize mice with tumor cells and to produce monoclonal antibodies that recognize melanoma cell surface molecules. Occasionally, monoclonal antibodies that recognize intracellular proteins as well are produced. With the use of this strategy, more than 40 different **melanoma-associated antigens** (MAAs) have been defined. These antigens can be grouped into several categories, including MHC molecules, growth factor receptors, cation binding proteins, high molecular weight extracellular matrix binding molecules, gangliosides, and nevomelanocytic differentiation antigens (see Table). Many of these antigens are expressed on normal cells of various types, but others are expressed only on cells in nevi or on normal or neoplastic cells of neural crest origin. Several of the high molecular weight, substrate interacting surface molecules are considered to be oncofetal antigens, since their expression is usually limited to developing tissues. Although antibodies to a few MAAs are detected in the serum of melanoma patients, there is no evidence that immune responses to these antigens play any role in protective immunity against the tumors.

Monoclonal antibodies specific for MAAs are used for three major purposes.

First, the biology of tumor progression has been studied by comparing the patterns of antigen expression on cells representing different stages in melanoma development, including melanocytes, nevus cells, *in situ* melanomas, and metastatic lesions. Correlation of the functional properties of these molecules with the growth phenotype of the cells on which they are expressed may help to elucidate mechanisms of tumor progression. For example, expression of melanoma ganglioside 2 (GD2) is restricted to advanced *in situ* and metastatic melanoma cells and GD2 is implicated as a cell adhesion molecule. Interestingly, GD2 is perhaps the most immunogenic MAA defined.

Second, monoclonal antibodies to MAAs are used for immunodiagnostic purposes, by immunohistochemical detection of MAAs on biopsy sections and serodiagnosis of melanoma based on detection of shed MAAs in the blood. Radioactive imaging of metastatic melanoma lesions in patients has been undertaken using radioactively labeled antibodies to melanotransferrin, chondroitin sulfate proteogylcan, and other MAAs.

Third, monoclonal antibodies against MAAs have been tried for immunotherapy. Unconjugated antibodies specific for gangliosides have been administered to melanoma patients, with limited success. Immunotoxins specific for MAAs are being evaluated in animal models. The strategies and limitations of antibody immunotherapy for tumors are discussed in the text.

Continued

Melanoma-Associated Antigens

Category	Example	Biochemical Characteristics	Significant Features
High molecular weight substrate interacting antigens	Chondroitin sulfate proteoglycan	>400 kD; 250 kD polypeptide core	Expressed on membrane spikes; involved in intercellular adhesion, and matrix attachment; highly immunogenic
	Melanoma-associated cellular adhesion molecule	105 and 130 kD	Role in matrix adhesion
	Placental membrane antigen	120 and 94 kD	Role in matrix adhesion
	High molecular weight proteins with ganglioside-like distribution pattern	260 kD	Role in matrix adhesion; highly specific to melanomas
Gangliosides	GD2 9-O-acetylated GD3 GD3 GM2	Gangliosides	Expressed in brain and tumors of neural crest origin only; implicated in cell adhesion; GD2 and GD3 expression characteristic of advanced or metastatic lesions; highly immunogenic
Growth factor receptors	Epidermal growth factor receptor (EGF-R)		Expressed on advanced tumors; EGF is mitogenic for melanoma cells *in vitro*
	Nerve growth factor receptor (NGF-R)		Expressed on all cultured melanoma cells
	Insulin growth factor receptor		
	Platelet-derived growth factor receptor (PDGF-R)		
	Transforming growth factor β receptor (TGF-β-R)		
Cation transport and binding proteins	Melanotransferrin	97 kD monomeric sialoglycoprotein; related to transferrin	Expressed on all cultured melanoma cells; highly immunogenic
	Calcium-binding S-100	21 kD highly acidic cytoplasmic protein	Member of calcium-binding protein family; expressed by neural crest-derived tumors and normal tissues; widely used for immunohistochemical diagnosis of nonpigmented melanomas.
Class II MHC	HLA-DR		Expressed on many primary tumor explants; no correlation with behavior *in vivo*
ICAM-1/2	—	90 kD	Ligands for LFA-1
Pigmentation-associated antigen		70-80 kD	Found in melanosomes of pigmented normal and malignant melanocytes
Differentiation antigens	Nevus antigen Gangliosides Galactocerebrosides Myelin-associated glycoprotein Others	Variable	Antigens on melanoma cells which correspond to antigens expressed on normal nevomelanocytes

Abbreviations: HLA, human leukocyte antigen; kD, kilodalton; MHC, major histocompatibility complex; GD, ganglioside; ICAM-1/2, intercellular adhesion molecule-1/2; LFA-1, leukocyte function-associated antigen-1.

pressed on developing (fetal) but not adult tissues. The expression of these proteins on tumor cells is the result of the derepression of genes by unknown mechanisms. As techniques for detecting these antigens have improved in sensitivity, it has become clear that their expression in adults is not strictly limited to tumors. These proteins are found in tissues in various inflammatory conditions, and even in small quantities in normal tissues. Furthermore, oncofetal antigens are not antigenic in the host, since they are expressed as self proteins during development. Not surprisingly, therefore, no evidence exists indicating that an individual mounts an immune response to these antigens on tumor cells. Nonetheless, the study of oncofetal antigens is useful for diagnostic purposes and provides some insights into tumor biology. The two most thoroughly described oncofetal antigens are **alpha-fetoprotein** (AFP), and **carcinoembryonic antigen** (CEA).

AFP is a 70 kilodalton (kD) α-globulin glycopro-

tein normally synthesized and secreted in fetal life by the yolk sac and liver. Fetal serum concentrations can be as high as 2 to 3 mg/ml, but in adult life the protein is replaced by albumin and only low levels are present in the serum. Serum levels of AFP can be significantly elevated in patients with hepatocellular carcinoma, germ cell tumors, and occasionally gastric and pancreatic cancers. Elevated serum AFP is a useful indicator of advanced liver or germ cell tumors or of recurrence of these tumors after treatment. Furthermore, the detection of AFP in tissue sections by immunohistochemical techniques can help in the pathologic identification of tumor cells. The diagnostic value of AFP as a tumor marker is limited by the fact that elevated serum levels are also found in non-neoplastic liver diseases such as cirrhosis.

CEA is a highly glycosylated 180 kD integral membrane protein that is a member of the Ig gene superfamily. CEA is also released into the extracellular fluid. Normally, high CEA expression is restricted to the gut, pancreas, and liver during the first two trimesters of gestation and reduced expression is found in normal adult colonic mucosa and lactating breast. CEA expression is greatly increased in colonic carcinomas, resulting in a rise in serum levels. Assays for serum CEA are used to monitor the spread of colon carcinoma or its recurrence after primary treatment. Recent studies have demonstrated that CEA functions as an intercellular adhesion molecule, promoting CEA-expressing cells to bind to one another. Thus, CEA may play a role in the way tumor cells interact with one another and with the tissues into which they are growing.

ANTIGENS ENCODED BY GENOMES OF ONCOGENIC VIRUSES

Viral antigens represent the most immunogenic molecules on malignant tumors and may be the most significant type of tumor antigens for protective tumor immunity. Both RNA and DNA viruses are implicated in the development of tumors in both experimental animals and humans. Virally induced tumors usually contain integrated proviral genomes in their cellular genomes and often express viral genome-encoded proteins. These endogenously synthesized proteins can be processed, and complexes of processed viral peptides with MHC molecules (usually class I) may be expressed on the tumor cell surfaces. Thus, tumor cells expressing viral proteins can stimulate and/or become the targets of specific T cell immune responses. Structurally and biologically distinct antigens are produced by various DNA and RNA tumor viruses.

DNA viruses are probably involved in the development of several different tumors. The papova viruses (including polyoma virus and SV40) and the adenoviruses induce a variety of malignant tumors in neonatal or immunodeficient adult rodents. Several different genes in these viruses cooperate to cause malignant transformation of infected cells. In humans, DNA viruses are associated with the development of several different tumor types. Examples include the association between Epstein-Barr virus (EBV) and B cell lymphomas (Box 17-2), human papilloma virus (HPV) and cervical carcinoma, and hepatitis B virus (HBV) and hepatocellular carcinoma. The viral genes responsible for producing the malignant phenotype in these human tumors are not well defined.

In most cases, DNA virus-induced tumor cells do not produce viral particles, and virally encoded protein antigens that are not components of infectious viral particles may be found in the nucleus, cytoplasm, or plasma membrane of the tumor cells. Specific immunity to DNA virus-encoded nuclear antigens protects against tumor development in animals. For example, SV40-induced tumors in mice express antigens that induce specific protective immunity against subsequent challenge with other SV40-induced tumors, but not against tumors induced by other viruses. Because these antigens are targets for tumor transplant rejection, they are functionally defined as TSTAs, as are the TSTAs of chemically induced tumors described earlier. The viral TSTAs, however, are not unique for each tumor but are shared by all tumors induced by the same type of virus (Table 17-1).

Different effector mechanisms mediate rejection of DNA virus-induced tumors, and different viral antigens serve as the immunologic targets. For example, the T antigen is a virally encoded nuclear protein expressed in SV40 transformed cells. The T antigen is required to produce the malignant phenotype, and it is not part of infectious virus particles. Immunization of experimental animals with this protein induces protective immunity against the development of SV40-induced tumors, and this immunity is mediated by class I MHC-restricted CTLs. Human adenovirus-induced rodent tumors express a virally encoded protein called E1A, which is found largely in the nucleus and is the principal determinant of the transformed phenotype of the infected cells. E1A is not part of infectious adenovirus particles. When class I-restricted CTLs specific for a processed peptide derivative of the E1A protein are adoptively transferred into mice with adenovirus-induced tumors, these CTLs kill the tumors (Fig. 17-3). There is no comparably well characterized DNA virus-encoded tumor antigen that is known to induce protective immunity in human tumors.

Both humoral and cell-mediated immune responses to DNA virus-encoded proteins expressed on tumor cells are clearly demonstrable in animals and humans. A protective role of the immune system in controlling the growth of DNA virus-induced tumors is suggested by the higher frequency of these tumors in immunodeficient individuals. In humans, EBV-associated lymphomas and HPV-associated skin cancers arise much more frequently in immunosuppressed individuals, such as allograft recipients receiving immunosuppressive therapy and acquired immunodeficiency syndrome (AIDS) patients, than in normal individuals. Adenovirus infection induces tumors much more frequently in neonatal or nude (congenitally T cell-deficient) mice, compared with nor-

BOX 17–2. THE RELATIONSHIPS BETWEEN EPSTEIN-BARR VIRUS, MALIGNANCY, AND IMMUNODEFICIENCY

Epstein-Barr virus is a double-stranded DNA virus of the herpesvirus family. The virus is transmitted by saliva, infects nasopharyngeal epithelial cells and B lymphocytes, and is ubiquitous in human populations worldwide. It infects human B cells by binding specifically to the type 2 complement receptor (CR2), followed by receptor-mediated endocytosis. Two types of cellular infections can occur. In a lytic infection, viral DNA, RNA, and protein synthesis begin, followed by assembly of viral particles and lysis of the host cell. Alternatively, a latent non-lytic infection can occur, in which the viral DNA is incorporated into the host genome indefinitely. Various virally encoded antigens are detectable in infected cells. **Epstein-Barr nuclear antigens** (EBNAs) include at least four different nuclear proteins that are expressed early in lytic infections and may also be expressed by some latently infected cells. EBNAs are the only well-characterized EBV antigens that have been shown to be targets for specific CTLs. Other viral structural protein antigens are expressed within infected cells and on released viral particles during lytic infections, including **viral capsid antigens** (VCAs). Antibodies specific for VCAs are present in acutely infected, recovering, and remotely infected individuals. Epstein-Barr virus has profound effects on B lymphocyte growth characteristics *in vitro*. First, the virus is a potent, T cell–independent polyclonal activator of B cell proliferation. Second, EBV can immortalize normal human B cells so that they will proliferate in culture indefinitely. The resulting long-term B lymphoblastoid cell lines are latently infected with the virus and may express EBNA proteins, but they do not have a malignant phenotype. The molecular basis for these effects of EBV on B cells is presently unknown.

There is a wide spectrum of sequelae to infection by EBV. Most people are infected during childhood, they do not experience any symptoms, and viral replication is apparently controlled by humoral and T cell–mediated immune responses. In previously uninfected young adults, **infectious mononucleosis** typically develops upon EBV infection. This disease is characterized by sore throat, fever, and generalized lymphadenopathy. Large morphologically atypical T cells are abundant in the peripheral blood of infectious mononucleosis patients. These cells are activated CTLs with specificity for EBV-encoded antigens. Previously infected, healthy individuals harbor the virus for the rest of their lives in latently infected B cells and, perhaps, in nasopharyngeal epithelium. It is estimated that one of every million B cells in a previously infected individual is latently infected. EBV infection is also strongly implicated as one of the etiologic factors for the development of certain malignancies, including nasopharyngeal carcinoma in Chinese populations, Burkitt's lymphoma in Equatorial Africa, and histologically variable B cell lymphomas in immunosuppressed patients.

There is compelling evidence that T cell–mediated immunity is required for control of EBV infections and, in particular, for the killing of EBV-infected B cells. First, individuals with deficiencies in T cell–mediated immunity often have uncontrolled, widely disseminated, and perhaps lethal acute EBV infections. Second, EBV-infected B cells isolated from patients with infectious mononucleosis can be propagated *in vitro* indefinitely, but only if the patient's T cells are thoroughly removed or inactivated by drugs such as cyclosporin A. In fact, immortalization of normal peripheral blood B cells by *in vitro* infection with EBV is usually successful only if the donor's T cells are removed or inactivated. Third, CTLs specific for EBV-encoded antigens are present in acutely infected and completely recovered infectious mononucleosis patients. Cloned CTL lines have been established *in vitro* that specifically lyse EBV-infected B cells, and these CTLs most often recognize peptide fragments of EBNA proteins in association with class I MHC molecules. It is possible that EBV-specific T cells are required *in vivo* to limit the polyclonal proliferation of infected B cells as well as to kill potentially immortalized clones of latently infected B cells. A loss of normal T cell–mediated immunity may allow latently infected B cells to progress toward malignant transformation. We discuss this hypothesis below.

The epidemiology and molecular genetics of Burkitt's lymphoma and other EBV-associated lymphomas have been the subject of intense investigation, and they offer fascinating insights into various aspects of viral oncogenesis and tumor immunity. Burkitt's lymphoma refers to a histologic type of malignant B cell tumor composed of monotonous small, malignant B cells. The African form of the disease is endemic in regions where both EBV and malaria infection are common. In these regions, the tumor occurs frequently in young children, often beginning in the jaw. Virtually 100 per cent of African Burkitt's lymphoma patients have evidence of prior EBV infection, and their tumors almost all carry the EBV genome and express EBV-encoded antigens. Malarial infections in this population are known to cause T cell immunodeficiencies, and this may be the link between EBV infection and the development of lymphoma. Sporadic Burkitt's lymphoma occurs less frequently in other parts of the world, and although these B cell tumors are histologically similar to the endemic form, only approximately 20 per cent carry the EBV genome. Both endemic and sporadic Burkitt's lymphoma cells have reciprocal chromosomal translocations involving immunoglobulin (Ig) gene loci and the cellular *myc* gene on chromosome 8 (see Box 4–5, Chapter 4).

B cell lymphomas occur at a high frequency in T cell–immunodeficient individuals, including individuals with congenital immunodeficiencies, AIDS patients, and kidney or heart allograft recipients receiving immunosuppressive drugs. Only some of these tumors can be called Burkitt's lymphomas, based on histologic appearance. Regardless of histologic appearance, most of these tumors share with Burkitt's lymphoma one or both of the features described above, namely *myc* translocations to Ig loci and latent infection with EBV.

These observations can be synthesized into a hypothesis about the pathogenesis of EBV-associated B cell tumors. African children with malaria, allograft recipients, congenitally immunodeficient children, and AIDS patients all have deficiencies in normal T cell function. EBV infection proceeds unchecked in these individuals, and EBV-induced polyclonal proliferation of B cells is uncontrolled. This rapid, exuberant proliferation of B cells increases the chances of errors made by recombinases or isotype switching enzymes, resulting in a relatively high frequency of genetic translocations to Ig loci. If the translocation involves the *myc* gene, this gene becomes transcriptionally deregulated. The resulting abnormal expression of *myc* may be causally related to malignant transformation and outgrowth of a neoplastic clone of cells. It is possible that other genetic events are required as well. For example, the integrated EBV genome may contribute to the malignant phenotype in EBV-positive lymphomas. This proposed scheme predicts that early in their course, EBV-associated B cell tumors may be polyclonal, since they arise from a polyclonally stimulated population of normal B cells. Later, one or a few clones may obtain selective growth advantages, perhaps because of deregulation of *myc*. As a result, the polyclonal proliferation evolves into a monoclonal or oligoclonal tumor. In fact, this has been shown to be the case by Southern blot analysis of Ig gene rearrangements in EBV-positive B cell tumors from immunosuppressed patients.

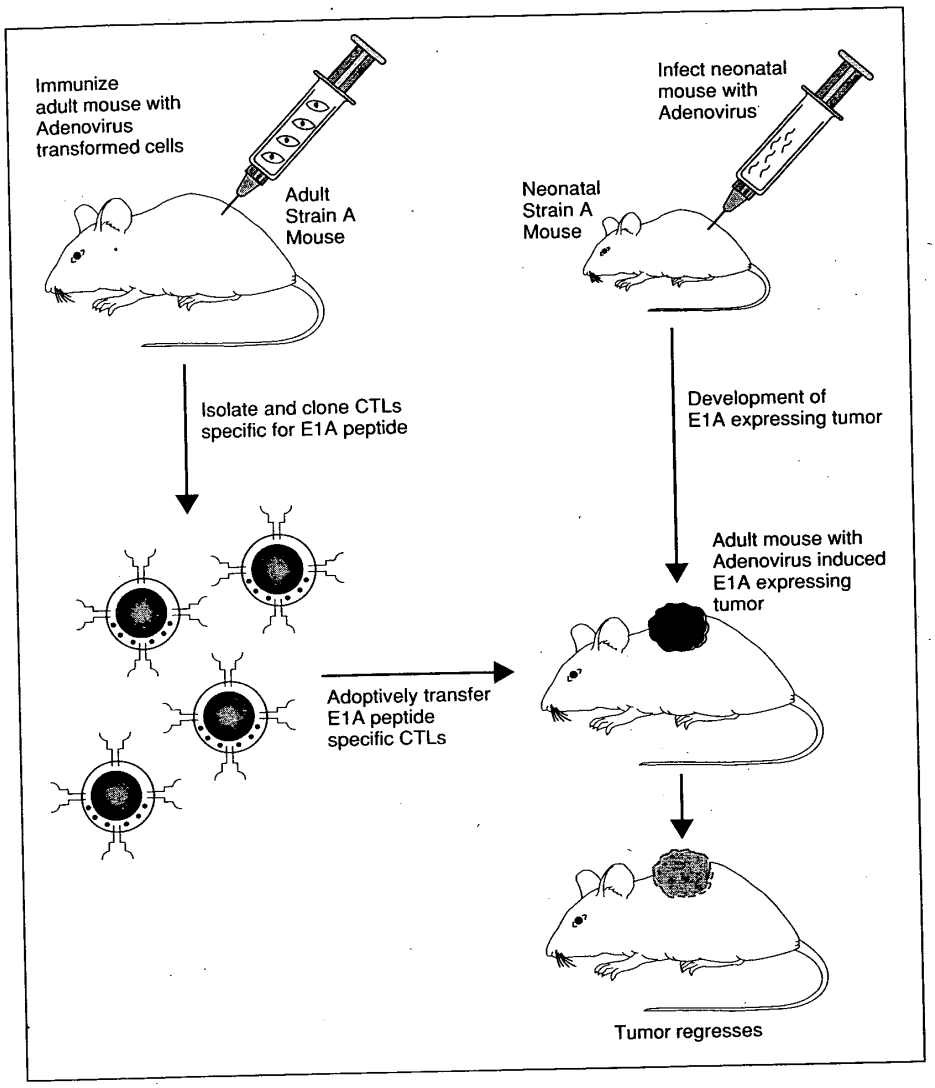

FIGURE 17–3. Viral antigen-specific cytolytic T lymphocytes (CTLs) kill virally infected tumors in vivo. *If neonatal mice are infected with adenovirus, they develop malignant tumors as adults and these tumors express the virally encoded E1A protein. The CTL clones isolated from a syngeneic mouse immunized with E1A-expressing cells can kill these E1A-expressing tumors when the CTLs are adoptively transferred to the tumor-bearing animal.*

mal adult mice. *Thus, a competent immune system may play a role in tumor immunosurveillance, not as a specialized anti-tumor function but because of its ability to recognize and kill virally infected cells.*

One of the clearest examples of viral oncogenesis is the development of tumors in animals infected with certain types of retroviruses (RNA tumor viruses). Some of these viruses carry well-defined oncogenes, induce tumors in days to weeks after infection, and are called acute transforming retroviruses. Examples of these acute transforming retroviruses include Rous sarcoma virus (carrying the *src* oncogene), avian myelocytomatosis virus (carrying the *myc* oncogene), and Kirsten murine sarcoma virus (carrying the v-K-*ras* oncogene). Other retroviruses, such as the murine leukemia viruses (MuLVs), cause tumors months after infection and do not carry any well-defined oncogenes. These slow-transforming retroviruses may cause tumors by inserting near, and deregulating transcription of, cellular genes that are responsible for growth control and differentiation.

The genomes of retroviruses are small, and there is a limited number of potentially immunogenic proteins that they may express in their host tumor cells. These proteins include products of the envelope *(env)* gene; core protein *(gag)* gene; and, in the case of acute transforming retroviruses, the oncogene. Retroviral oncogenes represent slightly altered forms of normal mammalian cellular genes, and therefore the viral oncogene products are usually not highly immunogenic. In contrast, humoral and cell-mediated immune responses to the *env* and *gag* products on tumor cells can be observed experimentally. Furthermore, *env* and *gag* products behave as TSTAs, stimulating CTL-mediated rejection of transplanted tumors. These TSTAs are shared by all tumors induced by the same type of retrovirus.

The only well-established human RNA tumor

virus is human T lymphotropic virus-1 (HTLV-1), which is the etiologic agent for adult T cell leukemia/lymphoma (ATL), an aggressive malignant tumor of $CD4^+$ T cells. Although immune responses specific for HTLV-1 encoded antigens have been demonstrated, it is not clear whether they play any role in protective immunity against development of tumors in virally infected people. Furthermore, ATL patients are often profoundly immunosuppressed, perhaps because of an effect of the virus on $CD4^+$ T cells, which the virus preferentially infects.

Tissue-Specific (Differentiation) Antigens on Tumor Cells

Tissue-specific, or differentiation, antigens are present on the surfaces of normal cells and are characteristic of a particular tissue type at a particular stage of normal differentiation of that tissue. Tumors that arise from a certain tissue often express the differentiation antigens of that tissue. Since these antigens are part of normal cells, they do not stimulate immune responses against the tumors on which they are expressed. The clinical significance of differentiation antigens on tumors relates to their use as targets for immunotherapy, discussed later, and also as diagnostic markers of the tissue of origin of tumors. The histologic appearance of a tumor may not be characteristic enough to permit a diagnosis of the type of normal tissue from which the tumor arose. Therefore, antibody probes for the expression of tissue-specific antigens may be required. For example, malignant lymphomas arising from the malignant transformation of a developing B cell may often be diagnosed as a B cell lineage tumor by the detection of a surface marker characteristic of normal pre-B cells, called CD10 (previously called common acute lymphocytic leukemia antigen, or CALLA). Tumors arising from more mature B cells are characterized by the presence of surface immunoglobulin. Examples of tissue-specific antigens expressed on tumors are listed in Table 17-2.

TABLE 17-2. Examples of Tissue-Specific Tumor Antigens Used in Clinicopathologic Analysis of Tumors

Tissue of Origin	Tumor	Antigens
B lymphocytes	B cell leukemias and lymphomas	CD10 (CALLA) Immunoglobulin
T lymphocytes	T cell leukemias and lymphomas	Interleukin-2 receptor (p55 chain) T cell receptor CD45R CD4/CD8
Prostate	Prostatic carcinoma	Prostate-specific antigen Prostatic acid-phosphatase
Neural crest-derived	Melanomas	S-100
Epithelial cells	Carcinomas	Cytokeratins

Abbreviations: CALLA, common acute lymphocytic leukemia antigen.

The Role of MHC Molecules in Anti-tumor Immunity

The expression of MHC proteins on tumor cells may be critical for immunologic recognition and destruction of the tumor cells. This is clearly the case if T cells are required for the cognitive and/or effector stages of specific anti-tumor immune responses, since T cells can recognize antigens only in association with MHC molecules. It is possible, therefore, that tumors that stimulate protective immune responses express adequate amounts of MHC molecules whereas other tumors that are not immunogenic fail to express enough or any MHC molecules. However, when the level of MHC expression on a broad range of experimentally induced or human tumor cells is compared with the growth properties of those cells, no clear correlation exists. For example, metastatic tumors, which presumably have evaded immune attack, do not express, on the average, any more or less MHC proteins than non-metastatic tumors. Although extensive analysis of the role of MHC expression on tumor growth *in vivo* has not permitted us to make any broadly applicable conclusions, some experimental models have established the importance of the MHC in the immune response to certain virally induced tumors.

Resistance to induction of neoplasms by tumor viruses often correlates with MHC gene haplotypes in inbred animals. For example, some murine RNA tumor viruses induce tumors only in some inbred strains of mice and not others, suggesting a requirement that certain MHC alleles be expressed in order for an anti-tumor immune response to occur. Such an immune response may be largely specific for a particular viral protein. If the immunodominant peptide from that protein binds only to a particular class I MHC allele, that peptide will be immunogenic only in strains of mice that express the allele. Thus, only certain inbred strains of mice will mount a protective anti-tumor immune response against tumors expressing the viral antigen. This is an example of an immune response (Ir) gene effect linked to class I rather than class II MHC molecules.

A more direct experimental analysis of the effects of MHC expression on tumor growth *in vivo* has been performed using rat cells transformed *in vitro* with viral oncogenes and then transplanted into syngeneic immunocompetent rats. We have described previously that the adenovirus E1A gene product is a tumor antigen that serves as a target for class I-restricted CTLs. Rat cells transformed by the Ad12 strain of adenovirus readily grow into tumors when injected into animals. In contrast, Ad5 strain adenovirus-transformed cells are not tumorigenic. This difference is correlated with the profound suppression of class I MHC expression in cells transformed by

Ad12, but not Ad5, virus. The suppression of MHC expression is an effect of the adenovirus E1A oncogene, but neither the mechanism of suppression nor the molecular basis of the differences between Ad5 and Ad12 strains is well understood. When class I MHC expression on AD12-transformed cells is increased by γ-interferon (IFN-γ) treatment or by transfection of autonomously transcribed class I MHC genes, these cells acquire the same non-tumorigenic phenotype as their Ad5-transformed counterparts (Fig. 17-4). Thus, class I expression is apparently required for inhibition of tumor growth in this model, consistent with the finding that class I MHC-restricted CTLs mediate killing of adenovirus-induced tumors. However, differences in the *in vivo* growth of Ad5 versus Ad12 transformed cells may be influenced by other factors, besides class I MHC expression, such as resistance to lysis by NK cells.

EFFECTOR MECHANISMS IN ANTI-TUMOR IMMUNITY

Tumor antigens elicit both humoral and cell-mediated immune responses *in vivo*, and many immunologic effector mechanisms are capable of killing tumor cells *in vitro*. The challenge for tumor immunologists is to determine which, if any, of these effector mechanisms are important in protective immune responses

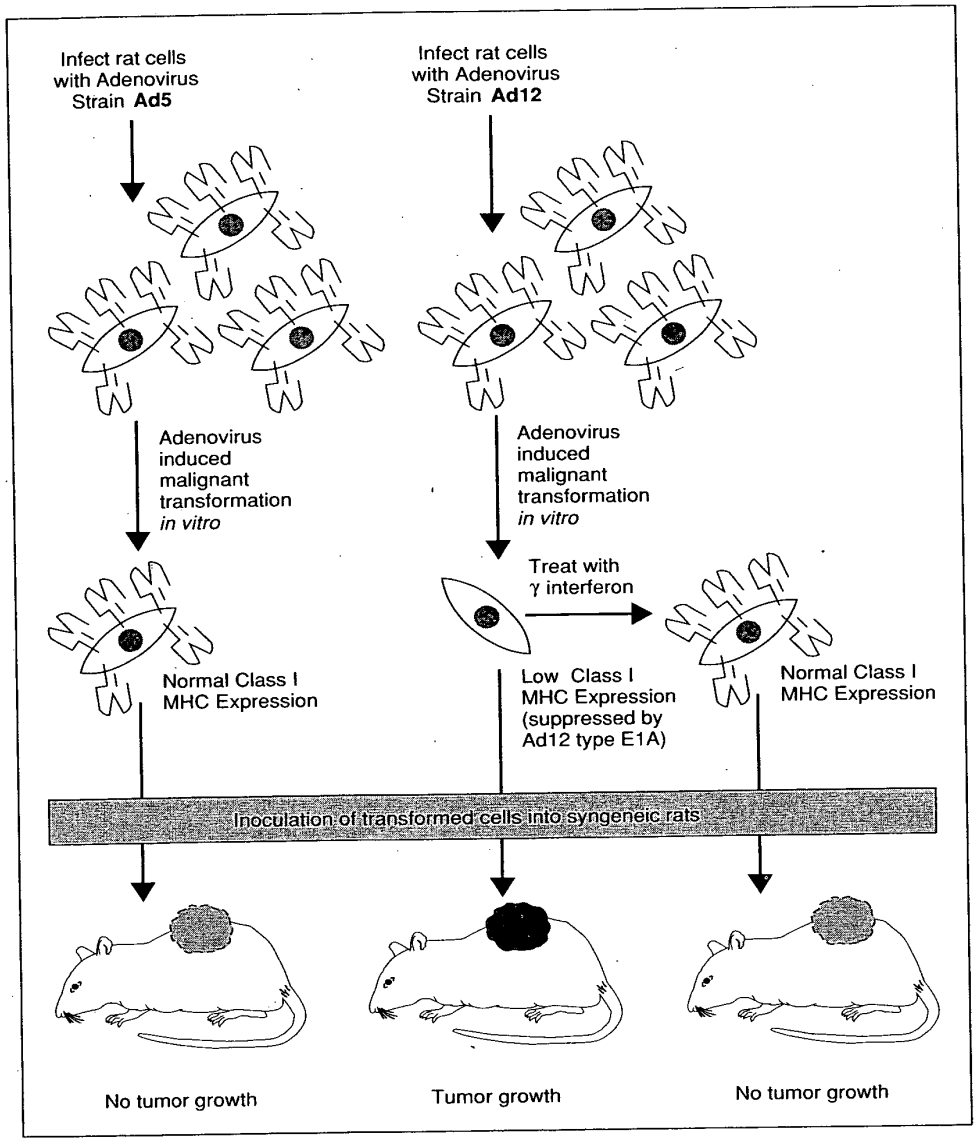

FIGURE 17-4. Relationship between class I MHC expression and tumorigenicity in adenovirus-induced tumors. *Rat cells that are malignantly transformed* in vitro *by infection with the Ad5 strain of adenovirus express normal levels of class I MHC molecules and are not tumorigenic in syngeneic rats. In contrast, rat cells that are malignantly transformed* in vitro *by infection with the Ad12 strain of adenovirus express low levels of class I MHC molecules and are tumorigenic. Ad12-infected tumors can be induced to express higher levels of class I MHC molecules by γ-interferon, and this treatment renders them non-tumorigenic. An interpretation of this experiment is that class I MHC expression on a virally induced tumor permits the host animal to mount a protective immune response, presumably against a virally encoded antigen presented by the tumor cell in association with class I MHC molecules.*

to spontaneously arising (non-experimental) tumors. In this section of the chapter, we briefly review the evidence for tumor killing by these various effector mechanisms and discuss which are the most likely to be relevant to human tumors.

Antibody Responses

Tumor-bearing hosts mount **antibody responses** specific for tumor antigens. The antigens that stimulate these immune responses are predictably limited to proteins that have not been expressed on normal tissues in a way that would induce tolerance. For example, as mentioned above, antibodies specific for the Tla are easily detected in normally Tla-negative mice bearing thymic leukemias. Patients with EBV-associated lymphomas have serum antibodies against EBV-encoded antigens expressed on the surface of their tumor cells. No evidence exists, however, for a role of such humoral responses in inhibiting tumor development or growth. A great variety of tumor cells can be lysed by antibody-dependent mechanisms *in vitro*. In these experimental situations, the antibodies against tumor surface proteins are often generated in other species and their tumoricidal activity is attributable to complement activation or to antibody-dependent cell-mediated cytotoxicity (ADCC) in which Fc receptor-bearing macrophages or NK cells mediate the killing. Whether or not these antibody-dependent mechanisms of tumor killing play a role *in vivo* remains unknown.

Cytolytic T Lymphocytes

CTLs provide effective anti-tumor immunity *in vivo*, as demonstrated in experimental tumor transplantation studies discussed earlier. In these cases, the effector cells are predominantly class I MHC-restricted CTLs which are fundamentally similar to virus specific or alloreactive CTLs described in Chapters 12 and 16. As discussed previously, the role for CTLs in immunosurveillance of non-virally induced tumors is questionable, since such tumors do not arise frequently in T cell-deficient animals or people or in patients with suppressed T cell immunity caused by therapeutic drugs or human immunodeficiency virus (HIV) infection. On the other hand, peripheral blood lymphocytes from patients with advanced tumors, including carcinomas and melanomas, contain CTLs that lyse explanted tumors from the same patients. Furthermore, mononuclear cells derived from the inflammatory infiltrate in human solid tumors, called tumor-infiltrating lymphocytes (TILs), also include CTLs with the capacity to lyse the tumor from which they were derived. However, the specificity of the anti-tumor CTLs derived from peripheral blood or tumors is not well established, since they often also show reactivity against unrelated tumor cells.

Natural Killer Cells

NK cells may be effector cells of natural and acquired immune responses to tumors. They utilize the same lytic mechanisms as CTLs to kill cells; however, they do not express T cell antigen receptors, and they kill targets in an MHC-unrestricted manner (see Chapter 12). NK cells lyse both virally infected cells and certain tumor cell lines, especially hematopoietic tumors, *in vitro*. In fact, lysis of such lines serves as a bioassay for NK activity. There appears to be a degree of specificity to NK killing, since many virally infected cells or tumor cells and most normal cells are not susceptible to NK lysis *in vitro*. The basis of this specificity is not understood. In addition, NK cells can be targeted to antibody-coated cells because they express low-affinity Fc receptors (CD16) for IgG molecules. The tumoricidal capacity of NK cells is increased by cytokines, including interferons, tumor necrosis factor (TNF), and interleukin-2 (IL-2). Therefore, their role in anti-tumor immunity may depend on the concurrent stimulation of T cells and macrophages which produce these cytokines. There is great interest in the role of IL-2 activated NK cells in tumor killing. These cells, called **lymphokine-activated killer (LAK) cells,** are derived *in vitro* by high-dose IL-2 treatment of peripheral blood cells or TILs from tumor patients. LAK cells exhibit a markedly enhanced and nonspecific capacity to lyse other cells, including tumor cells. The use of LAK cells in adoptive immunotherapy of tumors is discussed later.

A possible role for NK cells in tumor immunity *in vivo* is suggested by a variety of indirect evidence. For example, the incidence of tumors in different strains of inbred mice, or in mice of different ages, correlates inversely with the functional capacity of NK cells in these mice. Interestingly, T cell-deficient nude mice have normal or elevated numbers of NK cells and they do not have a high incidence of spontaneous tumors. Thus, it is possible that NK cells play a role in immunosurveillance against developing tumors, especially those expressing viral antigens. However, there is not a high degree of NK activity in the cellular infiltrates associated with solid human tumors, before *in vitro* expansion with IL-2.

Macrophages

Macrophages are potentially important cellular mediators of anti-tumor immunity. Their role is largely inferred from the demonstration that activated macrophages preferentially lyse tumor cells and not normal cells *in vitro*. The basis for this preferential susceptibility of tumor cells to macrophage mediated lysis is unknown. Like NK cells, macrophages express Fcγ receptors and they can be targeted to tumor cells coated with antibody. There are probably several mechanisms of macrophage killing of tumor target cells, some of which are essentially the same as the mechanisms of macrophage killing of infectious orga-

nisms. These include the release of lysosomal enzymes and reactive oxygen metabolites. Other reactive chemical species, such as nitric oxide, may also play a role.

Activated macrophages also secrete the cytokine TNF, which, as its name implies, was first characterized as an agent which can kill tumors but not normal cells. The various actions of TNF have been discussed in Chapter 11. There is convincing evidence that a major component of macrophage-mediated killing of tumors is due to TNF secretion. For example, tumor cells selected *in vitro* for resistance to killing by TNF are often also resistant to killing by macrophages. Killing by both mechanisms is slow (24 to 48 hours), is augmented by protein or RNA synthesis inhibitors, and involves nuclear DNA fragmentation rather than osmotic lysis.

TNF kills tumors by at least two different mechanisms. First, *binding of TNF to high-affinity cell surface receptors is directly toxic to tumor cells.* The toxicity may be a result of the production of free radicals. Normal cells respond to TNF by synthesizing superoxide dismutase, an enzyme that participates in the inactivation of free radicals. In contrast, many tumor cells fail to make superoxide dismutase in response to TNF. Thus, part of the explanation of selective tumor cell killing by TNF may be loss of responses in these cells that serve to protect normal cells. Direct toxic effects of TNF may also involve disruption of cytoskeletal proteins, or interference with gap junction formation. Second, *in vivo, TNF causes tumor necrosis by mobilizing various host responses.* In fact, even tumor cells lacking TNF receptors can be eradicated in mice by treatment with TNF. The key observation is that TNF selectively eradicates vascularized tumors and is much less effective in killing avascular implants. Histologically, the response to TNF, described as hemorrhagic necrosis, looks very much like the localized Shwartzman reaction described in Chapter 11. This resemblance has led to the suggestion that TNF acts selectively on tumor vessels to produce a Shwartzman-like reaction leading to thrombosis of the vessels and ischemic necrosis of tumors. Tumor vessels, unlike normal vessels, may be already "primed" to trigger the Shwartzman response once they encounter TNF. The differences between normal and tumor vessels are a subject of intense study.

MECHANISMS OF EVASION OF THE IMMUNE SYSTEM BY TUMORS

Although many malignant tumors are weakly immunogenic, there are numerous examples of tumor antigens that can stimulate strong immune responses. A major focus of tumor immunology is to understand the ways in which tumor cells evade immune destruction, despite their potential immunogenicity. The process of evasion, often called "tumor escape," may be a result of one or more mechanisms.

1. Some tumors may be poorly immunogenic in a particular host because the host does not express the appropriate MHC molecules necessary for binding and presenting processed derivatives of tumor antigens. This immune response gene effect is hypothesized to be the reason why some strains of mice are resistant to tumor induction by murine leukemia viruses whereas others are not.

2. MHC expression may be down-regulated on tumor cells so that they cannot form immunologically recognizable complexes of processed tumor antigens and MHC molecules. We have previously discussed the correlation of MHC down-regulation and tumorigenicity in adenovirus-induced tumors.

3. A host may be tolerant to some tumor antigens, either because of neonatal exposure to such antigens or because the tumor cell may present its antigens to the immune system in a tolerogenic form, e.g., in high doses or without the proper costimulators (see Chapter 10). Neonatally induced tolerance has been demonstrated for tumors caused by the murine mammary tumor virus. This virus causes breast tumors in adult mice that have acquired the viral infection by neonatal nursing. Although these tumors are not seen as foreign in these mice and do not stimulate an immune response, they are highly immunogenic when transplanted to syngeneic virus-free mice. Another example of neonatally induced tolerance to virally encoded tumor antigens is seen in SV40-transgenic mice. Strains of SV40-transgenic mice that express SV40 genes during early development and have a high incidence of tumors do not mount immune responses against the SV40 T antigen. In contrast, other SV40-transgenic mice that have a low incidence of tumors are immunologically reactive to the SV40 T antigen.

4. The kinetics of tumor growth may allow for the establishment of immunologically resistant tumors before an effective immune response develops. This phenomenon, called "sneaking through," has been experimentally modeled by transplantation studies. Transplantation of small numbers of tumor cells may lead to the establishment of lethal tumors, whereas larger transplants of the same tumor are rejected. One postulated reason for this apparent contradiction is that low doses of tumor antigens are not sufficiently stimulatory to the immune system, and by the time a large number of tumor cells grow in the transplant recipient, mutations in tumor antigen genes may have occurred that reduce the chance of immune recognition.

5. Anti-tumor immunity may result in selection of mutant tumor cells that have lost expression of immunogenic proteins, especially if such proteins are not critical for the malignant phenotype of the tumor. Given the generally high mitotic rate of tumor cells and their genetic instability, such mutations are theoretically likely. Analysis of tumors that are serially transplanted from one animal to another has shown that the loss of antigens recognized by tumor-specific CTL clones correlates with increased growth and metastatic potential.

6. The loss of surface expression of tumor antigens as a result of antibody binding, called **antigenic modulation,** leads to acquired resistance to immune effector mechanisms. Antigenic modulation is due to either endocytosis or shedding of the antigen-antibody complexes. Thus, some non-complement-fixing anti-tumor antibodies may protect tumor cells from other, complement-activating antibodies. Antigenic modulation is perhaps most relevant as a problem complicating attempted passive immunotherapy with anti-tumor antibodies.

7. Antigens shed by tumors, and complexes of antibody with shed tumor antigens, have been postulated in the past to act as blocking factors that interfere with immune responses to tumors. The mechanisms of action of blocking factors remain obscure but could involve functional blockade of NK cell Fc receptors, or induction of "suppressor cells" which specifically down-regulate the function of tumor antigen-specific helper T cells.

8. Tumor cell surface antigens may be hidden from the immune system by glycocalyx molecules, including sialic acid-containing mucopolysaccharides. This is called "antigen masking" and may be a consequence of the fact that tumor cells often express more of these glycocalyx molecules than do normal cells. Similarly, some tumors may shield themselves from the immune system by activating the coagulation system, thereby investing themselves in a "fibrin cocoon."

9. Immunosuppression may be induced by tumor products or by the chemical, physical, or infectious agents that induce malignant transformation of cells. An example of an immunosuppressive tumor product is transforming growth factor-β (TGF-β), which is secreted in large quantities by many tumors. TGF-β inhibits a wide variety of lymphocyte and macrophage functions (see Chapter 11). Immunosuppressive carcinogenic agents include ionizing radiation, chemotherapeutic agents, and certain viruses. These agents can kill or functionally inhibit lymphocytes.

IMMUNOTHERAPY OF TUMORS

The potential for treating cancer patients by immunologic approaches has held great promise for immunologists and cancer biologists over much of this century. Recent advances in our understanding of the immune system have encouraged a variety of new strategies. Next we describe some of the modes of tumor immunotherapy that have been tried in the past or are currently being investigated.

Stimulation of Immune Effectors

The development of virally induced tumors can be blocked by vaccination with viral antigens. This approach is successful in reducing the incidence of feline leukemia virus-induced hematologic malignancies in cats and in preventing the herpesvirus-induced lymphoma called Marek's disease in chickens. In humans, it is possible that the ongoing vaccination program against the hepatitis B virus may reduce the incidence of hepatocellular carcinoma, a cancer that is associated with HBV infection of the liver.

Many different approaches have been used for the immunotherapy of already established tumors. Nonspecific immune stimulation of tumor patients with adjuvants, such as the bacille Calmette-Guérin (BCG) mycobacterium injected at the sites of tumor growth, has been tried many times. This treatment serves predominantly to activate macrophages. Oncologists are still assessing the potential of local BCG administration in bladder carcinomas and melanomas. Another experimental approach to nonspecific immune stimulation is the administration of low doses of anti-CD3 antibodies to mice with transplanted fibrosarcomas. This treatment results in polyclonal activation of T cells and, concomitantly, prevention of tumor growth. The dose of anti-CD3 is of critical importance since, as described in Chapter 16, high doses of anti-CD3 antibody are widely used as an immunosuppressant to prevent allograft rejection.

Immunization of tumor-bearing hosts with tumor cells is an experimental approach previously attempted in experimental animals and in humans. Leukemic patients have been immunized with killed leukemic cells from other patients with little success. In attempts to make them more immunogenic, animal tumors have been altered by covalently linking haptens (such as trinitrophenol) to their surface or by infecting the tumor cells with viruses (such as vaccinia virus). These altered cells are then used to immunize tumor-bearing animals. The rationale for this approach is based on the assumption that immune responses to the altered tumor cells will then be effective on the unaltered tumor cells, although the basis for this cross-reaction is not clear. The results suggest that such procedures may enhance active anti-tumor immunity, but their feasibility in the clinical situation is unproven.

Antibody Therapies

There are many variations on the use of *antibodies specific for tumor antigens* in tumor therapy (Table 17-3). The theoretical potential of using tumor-specific antibodies as "magic bullets" remains alluring to many investigators:

1. *Anti-idiotypic antibodies* have been used in the treatment of B cell lymphomas expressing surface Ig with particular idiotypes. The idiotype is a highly specific tumor antigen, since it is expressed only on the neoplastic clone of B cells and on no other cells. (Anti-idiotypic antibodies are raised by immunizing rabbits with a patient's B cell tumor, and depleting the serum of reactivity against all other human immunoglobulins.) This strategy relies on complement fixation or ADCC in order for the lymphoma cells to be killed. The approach is not generally successful, and

TABLE 17-3. Examples of Immunotherapy with Anti-tumor Antibodies

Approach	Examples	Tumors	Current Status
Free antibody	Anti-Ig idiotype	B cell lymphomas	Human trials
	Anti-IL-2R	T cell lymphomas	Human trials
	Anti-ganglioside	Melanoma	Human trials
	Anti-*neu* oncogene product	Sarcoma	*In vivo* animal model
Ig-toxin conjugates	Ricin A-anti-CD5	T cell lymphomas	*In vitro*
	Ricin A-anti-CD22	B cell lymphomas	Human trials
	Ricin A-anti-CD19	B cell lymphomas	*In vitro*
	Ricin A-anti-melanoma	Melanoma	Human trials
Ig-drug conjugates	Chlorambucil-anti-melanoma	Melanoma	Human trials
Ig-radioisotope conjugates	^{211}Bismuth-anti-Thy-1	T cells	*In vitro*
Dual-specificity heteroconjugate Ig	Anti-CD3:Anti-TAA	Sarcoma	*In vitro*
Ig-hormone heteroconjugate	Anti-CD3: Melanocyte-stimulating hormone	Melanoma	*In vitro*

Abbreviations: Ig, immunoglobulin; IL-2R, interleukin-2 receptor; TAA, tumor-associated antigen.

there are many theoretical reasons why it may not work. Since surface Ig expression is not functionally related to the malignant phenotype of the cell, selective outgrowth of non-Ig-expressing tumor cells can occur. Alternatively, the high degree of somatic mutation known to occur in Ig genes could result in the selective outgrowth of tumor cells with altered idiotypes no longer reactive with the anti-idiotypic antibody. Furthermore, since the rabbit antibodies are foreign proteins, the tumor patient may develop anti-rabbit Ig antibodies and these may interfere with the efficacy of the rabbit-anti-tumor antibodies. Attempts to circumvent these problems by producing human monoclonal antibodies or by injecting cocktails of several different antibodies have also not proven successful.

2. *Antibodies directed against growth factor receptors (IL-2 receptors)* have been used in the experimental therapy of human T lymphocyte malignancies, including HTLV-1 associated leukemias and lymphomas. The rationale of this approach is that IL-2 may serve to stimulate the growth of these tumor cells, and such antibodies may cause modulation or functional blockade of IL-2 receptors (IL-2R). Alternatively, such antibodies could cause complement-mediated lysis of the IL-2R expressing tumor cells. Anti-IL-2R therapy is not tumor specific and may be immunosuppressive because normal T cells would be rendered nonfunctional. There is also no evidence to support an obligatory role of IL-2 as an autocrine or paracrine growth factor for HTLV-1-induced tumors *in vivo*. Although initial trials have met with little success, anti-growth factor receptor antibodies could theoretically be useful for the treatment of other tumors.

3. *Antibodies specific for an oncogene product* might be able to inhibit tumor growth if that oncogene product is essential for the transformed phenotype. Monoclonal antibodies against the *neu* oncogene encoded cell surface protein cause *neu*-transformed cells to revert to a nontransformed phenotype *in vitro*, and the same antibodies can inhibit tumor growth in mice.

4. *Anti-tumor antibodies coupled to toxic molecules, radioisotopes, and drugs* are being used in immunotherapy trials in cancer patients and in experimental animals. Toxins such as ricin or diphtheria toxin are highly potent inhibitors of protein synthesis and are theoretically useful at extremely low doses if they are bound to tumor-specific antibodies to form **immunotoxins.** This approach requires the covalent attachment of the toxin to an antibody molecule without loss of toxicity or antibody specificity. Furthermore, the immunotoxin must be endocytosed and delivered to the appropriate intracellular site of action. Another approach is to covalently attach anti-neoplastic drugs or cytocidal radioisotopes to anti-tumor antibodies. Two practical difficulties must be overcome for this technique to be successful. First, the specificity of the antibody must be such that there is not significant binding to non-tumor cells. As we have discussed, there are few truly tumor-specific antigens to select when designing an antibody-based immunotherapy approach. Second, it may be difficult to ensure that a sufficient amount of antibody reaches the appropriate target, before clearance of the antibody by Fc receptor-bearing phagocytic cells. Such clearance may not only reduce anti-tumor effectiveness, but also may damage phagocytic cells. $F(ab')_2$ conjugated toxins may minimize the latter problem.

5. *Heteroconjugate antibodies* may allow targeting of cytotoxic effector cells onto tumor cells. In this approach, an antibody specific for a tumor antigen is covalently coupled to an antibody directed against a surface protein on cytotoxic effector cells, such as NK cells or CTLs. Such heteroconjugates promote binding of the NK cells or CTLs to appropriate tumor targets. A heteroconjugate consisting of an anti-CD3 antibody coupled to an antibody against a tumor cell surface protein enhances CTL-mediated lysis of the tumor

cell. In this case, the anti-CD3 antibody serves not only to bring the CTL into proximity of the target cell but also to activate the CTL.

6. *Conjugates of antibodies and hormones* can be used to target CTLs to tumor cells expressing hormone receptors. For example, anti-CD3 antibodies coupled to melanocyte-stimulating hormone enhance *in vitro* destruction of hormone-binding human melanoma cells by CTLs. A related (but not strictly immunologic) strategy is to generate fusion proteins with toxic activity and tumor-binding capacity. This is done by engineering and expressing genetic constructs in which bacterial toxin genes are linked to the genes encoding the binding domains of hormones or other ligands that bind to the tumor cells. Fusion proteins combining IL-2 and protein toxins have been used as T cell lytic agents in experimental transplantation (see Chapter 16).

7. *In vitro depletion of bone marrow tumor cells by antibody plus complement-mediated lysis* is used in autologous bone marrow transplants in B cell lymphoma patients. In this protocol, some of the patient's bone marrow is removed and the patient is given lethal doses of irradiation and chemotherapy, which destroy tumor cells. This treatment also destroys the remaining normal marrow cells in the patient. The bone marrow that was removed earlier is then treated with antibodies directed against B lymphocyte-specific antigens, which are expressed on the B cell-derived lymphoma cells. Complement is then added to promote lysis of the lymphoma cells that have bound antibody. The bone marrow, having been purged of lymphoma cells, is reinjected into the patient to reconstitute the hematopoietic system destroyed by irradiation and chemotherapy.

Adoptive Cellular Immunotherapy

Adoptive cellular immunotherapy refers to the transfer of cultured immune cells that have anti-tumor reactivity into a tumor-bearing host. Two variations to this approach are currently in clinical trials:

1. *Lymphokine-activated killer cell therapy* involves the *in vitro* generation of LAK cells by culturing peripheral blood leukocytes removed from tumor patients in high concentrations of IL-2. The LAK cells are then injected back into the cancer patient. As we discussed previously, LAK cells are derived mainly from NK cells. Adoptive therapy with autologous LAK cells, in conjunction with *in vivo* administration of IL-2 or chemotherapeutic drugs, has had impressive results in mice, with regression of solid tumors. Human LAK therapy trials have so far been largely restricted to advanced cases of metastatic tumors, and the efficacy of this approach cannot yet be fully evaluated.

2. *Tumor-infiltrating lymphocyte therapy* involves the generation of LAK cells from mononuclear cells originally derived from the inflammatory infiltrate present in and around solid tumors, obtained from surgical resection specimens. The rationale for this approach is that TILs may be enriched for tumor-specific killer cells. In fact, TILs include activated NK cells and CTLs, both of which appear to kill cells nonspecifically. High doses of IL-2 may impart CTLs with the capacity to kill targets without specific T cell receptor (TCR)-mediated binding. Human trials with TIL therapy are ongoing.

Cytokine Therapy

Cytokines are also used for the treatment of various tumors. This type of experimental therapy has become feasible only recently with the production of highly purified or recombinant cytokines in sufficient quantities. The rationale for using cytokines is based on their ability to enhance one or more components of cellular immune function; the effects of the cytokines are not specific for anti-tumor-directed immune effector cells.

1. IL-2, administered in high doses, is being used alone or in conjunction with adoptive cellular immunotherapy. This treatment is effective in inducing measurable tumor regression in 20 to 40 per cent of patients with melanoma and renal cell carcinoma. Presumably, the IL-2 works by activating NK cells and/or CTLs, i.e., inducing LAK cell differentiation *in vivo*. The treatment can be highly toxic, causing fever, pulmonary edema, and often shock. These toxic effects are probably indirectly mediated by IL-2 acting on other lymphocytes to enhance production of TNF, IFN-γ, and lymphotoxin. Interleukin-4 (IL-4) also can activate CTLs and is currently being tested in clinical trials as an alternative agent with potentially fewer side effects.

2. TNF has been used in preliminary cancer treatment protocols in patients with advanced carcinomas. Although TNF clearly has potent anti-tumor effects *in vitro*, it has many undesirable pathologic effects and can be highly toxic at the doses that are required for tumor killing *in vivo*.

3. Alpha-interferon (IFN-α) is a type I interferon, produced largely by leukocytes (see Chapter 11). It has antiproliferative effects on cells *in vitro*, increases the lytic potential of NK cells, and increases class I MHC expression on various cell types. This cytokine has been used in extensive clinical trials, with promising results. Objective tumor regression responses occur in 10 to 15 per cent of renal cell carcinomas, melanomas, and Kaposi sarcomas; 40 to 50 per cent of various lymphomas; and 80 to 90 per cent of hairy cell leukemias (a B cell lineage tumor). In fact, IFN-α treatment of hairy cell leukemia is now standard practice and is currently the only reliable cytokine therapy for a human cancer.

4. IFN-γ has been used in clinical trials for the treatment of various hematopoietic and solid tumors, with little success. It was hoped that the macrophage and NK cell-activating properties of this cytokine, as

well as its ability to up-regulate MHC molecule expression, would help to enhance anti-tumor immunity. Intraperitoneal administration of IFN-γ for the treatment of ovarian carcinomas is currently being evaluated.

5. Hematopoietic growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are used in cancer treatment protocols, although not strictly to enhance immune responses against tumors. Rather, they shorten periods of neutropenia following chemotherapy or after autologous bone marrow transplantation by stimulating the maturation of granulocyte precursors.

An interesting experimental approach to cytokine treatment of tumors is the transfection of tumor cells *in vitro* with cytokine genes followed by transplantation of the cells into tumor-bearing animals. In this way, immunostimulatory cytokines are produced in abundance specifically at the site of tumor growth. This has been accomplished in different animal tumors with IL-2, IL-4, and IFN-γ genes. In each case, transfection of the cytokine gene inhibits tumor growth *in vivo* and in each case the inhibition is due to stimulation of a different immune effector mechanism by the secreted cytokine. For example, IL-4 transfected tumor cells stimulate an intense eosinophilic inflammatory response *in vivo* and do not grow into lethal tumors and IL-2 transfected colon carcinoma cells stimulate a protective CTL response in mice. The potential of this type of strategy for treatment of human tumors remains hypothetical.

SUMMARY

Malignant tumors express a variety of antigens that may stimulate and serve as targets for anti-tumor immunity. Protective anti-tumor immune responses have been convincingly demonstrated in experimental animal models. It has been more difficult to demonstrate that natural or acquired immune responses to most common human tumors serve to control their development or growth, but this may reflect the limitations of analysis of immune responses in humans. The development of tumors induced by viruses, which express virally encoded antigens, is likely to be inhibited by specific immune responses. Antigens unique to individual tumors, which stimulate specific rejection responses upon transplantation, have been demonstrated only in experimental animal tumors. Other tumor antigens that can stimulate immune responses are shared by different tumors, and these include viral antigens and products of derepressed genes. Tumors may also express tissue differentiation antigens or embryonic antigens to which the host is tolerant; these molecules are useful diagnostic markers. MHC molecule expression may vary from tumor to tumor; in some tumors, MHC expression may be necessary for protective immune responses.

Virtually every immunologic effector mechanism known can destroy tumor cells *in vitro*. One or more of these mechanisms may work on tumor cells *in vivo*, and different mechanisms may be effective on different tumors. Natural killer cells, CTLs and macrophages are probably the major effectors of anti-tumor immunity *in vivo*. Various mechanisms have been proposed to explain how potentially immunogenic tumors escape destruction by the immune system. These mechanisms include MHC-linked genetic unresponsiveness of the host, down-regulation of MHC molecules, induction of tolerance to tumor antigens, loss of expression of immunogenic proteins due to mutations, modulation of tumor antigens by anti-tumor antibodies, antigen masking by extracellular proteins, and immunosuppression of the host. A variety of immunologic approaches for treating cancers, including anti-tumor antibodies, adoptive cellular immunotherapy, and cytokine treatment are currently in clinical trials.

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CHAPTER 24

Idiotypic Networks

John F. Kearney

NATURE OF IDIOTYPES AND ANTI-IDIOTYPES

It was first shown by Kunkel et al. (1) and Oudin and Michel (2) that immunoglobulins contained both common and individual antigenic determinants. Thus heterologous antisera produced by immunization with one monoclonal immunoglobulin (or a restricted set of immunoglobulins) could be absorbed extensively against other antibodies of the same light and heavy chain isotype and would then react only with the immunizing immunoglobulin. The antigen(s) recognized by such sera was defined as the idiotype(s) that was correctly predicted to be contained in the variable (V) regions of the immunoglobulin(s) used for immunization. As seen in Chapter 9, the diversity of variable regions generated during VDJ_H and VJ_L recombination and by somatic mutation would permit the expression of large numbers of potential idiotypes. Since the term idiotype defines a collection of light and/or heavy chain V-region-associated structures, which can involve any part of the exposed V regions, certain structures, because of the homologies among V regions will be shared by different immunoglobulin molecules. These idiotypes can be expressed by antibodies with different antigenic specificities as first described by Oudin and Cazenave (3). The ability to raise monoclonal anti-idiotypic antibodies has led to the revision of the original definition of an idiotype as a rarely expressed determinant on immunoglobulins. A monoclonal anti-idiotype antibody may detect a single determinant that is shared even more widely than the sum of the set of determinants detected by conventional heterologous anti-idiotype antibody. Thus in conventional direct binding or binding inhibition assays, a heterogeneous purified anti-idiotypic antibody preparation would appear to be more specific than a monoclonal anti-idiotypic antibody by binding to fewer Ig molecules, at least as detected by radio immunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) (4). In contemporary terms, "idiotope" is used to define V-region-associated structures that are detected using monoclonal anti-idiotypic antibodies. It follows then that an idiotype of a given immunoglobulin molecule can be described as a collection of idiotopes as mapped by a panel of monoclonal anti-idiotopic antibodies or a conventional cross-absorbed polyclonal anti-idiotype antibody.

If the topography of a given idiotype as detected by an antibody preparation occurs at a high frequency on a given population of immunoglobulins (or Ig receptors on B cells), the Oudin–Cazenave phenomenon will occur to a greater or lesser extent; for example, some determinants expressed by a particular V_H or V_L region that does not depend on the restricted association of any par-

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ticular V_H with any particular V_L, respectively, may be expressed at a high frequency on immunoglobulins not related to the specificity of the immunizing antibody. These kinds of exclusively V_H- or V_L-associated idiotopes are not frequently detected although there are notable exceptions (5). Furthermore, there are well-documented examples of particular idiotopes that are hapten inhibitable (and therefore binding site related) and that are expressed only when these V regions are associated with particular heavy chain constant regions. Such examples demonstrate that constant region domains can also influence V region structure and topography (6–8).

The recent understanding of V region genetics and structure, as aided by recombinant DNA and crystallographic techniques, has allowed a limited mapping of the three-dimensional structures that constitute an idiotope detected using monoclonal anti-idiotypic antibodies. However, as shown later, this process is difficult. The use of an idiotype as a clonal marker for B cells using anti-idiotypic antibodies is limited because in many cases multiple related or even nonrelated clones may be detected by a given antibody, depending on the degree of sharing of idiotopes among different Ig molecules. Furthermore, it has been demonstrated that certain public idiotypes may be shared between strains, or individual humans, or even species (9–11).

Operationally, the definition of idiotype and anti-idiotype has changed from the original concept of Kunkel and Oudin. Nevertheless, anti-idiotypic antibodies have been of great importance in the definition of related families of immunoglobulin and Ig-producing B cell precursors. While an in-depth discussion of the construction and specificity analysis of anti-idiotypic antibodies is not warranted in this chapter, some general principles are discussed.

Conventional anti-idiotype antibodies are made by intraspecies or interspecies immunization with a purified antigen-specific pool of antibodies or a monoclonal antibody. The resulting antiserum is then extensively absorbed against similar molecules with the same constant region to remove antibodies with anti-C_HC_L specificities. For a more detailed technical discussion the reader is referred to Briles et al. (4). These antisera can detect a range of topographies and idiotypes on different molecules. The production of anti-Id antibodies against self-idiotopes by Rodkey (12) was one of the first key predictions of the network theory. More recently, the use of hybridoma technology has facilitated the detection of idiotopes so that a given V region can be mapped serologically by the use of monoclonal antibodies (mAbs) as shown in the example in Fig. 1.

As described in Fig. 1, a set of mouse α1-3 dextran-specific antibodies (IgM,λ1) with known V region sequences (13) has been tested against several monoclonal anti-Id antibodies, in an attempt to determine the amino acid residues involved in construction of a given idiotope (14). The anti-DEX antibodies J558 and M104 differ only in the D segment, and the monoclonal anti-idiotypic antibodies (MAIDs) EB3-7 and SJL-18-1 clearly recognize this difference. In contrast, the antibody CD3-2 reacts with the entire panel of anti-DEX antibodies except HDEX8, where it shows only partial reactivity, and HDEX10, where there is no reactivity. Therefore the IdX (or cross-reactive determinant) detected by the monoclonal antibody CD3-2 appears to be defined within the HV2 region of the molecule, particularly the amino acids Asn-Asn normally expressed at positions 54 and 55. Reactivity is reduced when the Asn at 54 is replaced with a Lys, and it is abolished in HDEX10, where the amino acids in both positions are altered to Lys-Lys. In contrast, the D segments appear to be involved in determining the individual idiotypic determinant (IdI). It should be noted, however, that the mAb EB3-7 reacts not only with J558 but also with HDEX6. From these kinds of observations it can be concluded that D region residues influence the idiotype detected by these antibodies to a great degree. However, it is clear that antibodies with a different D region can also react.

Another elegant method for making a three-dimensional map of idiotypic determinants is by the use of transmission electron microscopy of negatively stained immune complexes. Roux et al. (15) have studied the interactions of rat monoclonal anti-Id antibodies to HGAC 30, a mouse IgG3,κ antibody specific for streptococcal group A carbohydrate. These studied showed that idiotopes, at least as defined by these anti-Id antibodies, were dispersed over the variable region domains extending from the complementarity-determining region (CDR) to the variable-constant switch region.

Taken together, these findings emphasize that monoclonal anti-idiotype antibodies cannot always be used as strict clonal markers because of the vast size and heterogeneity of the immunoglobulin repertoire. These expected cross-reactivities will appear operationally and may involve a larger pool of target Ig molecules that share the same idiotypic determinant, not all of which share the same antigen-binding specificity. These MAIDs may also react on occasions, not only with immunoglobulin V region, but with other nonimmunoglobulin structures present on B cells and other nonimmunoglobulin molecules not associated with lymphoid cells (16).

Most recently, idiotope loss variants have been used to establish amino acid sequence correlates that could be defined by the appropriate monoclonal anti-idiotype antibodies. These studies confirmed earlier studies and showed that even single amino acid substitutions could drastically alter idiotope expression with contributions from both light and heavy chains (17–19).

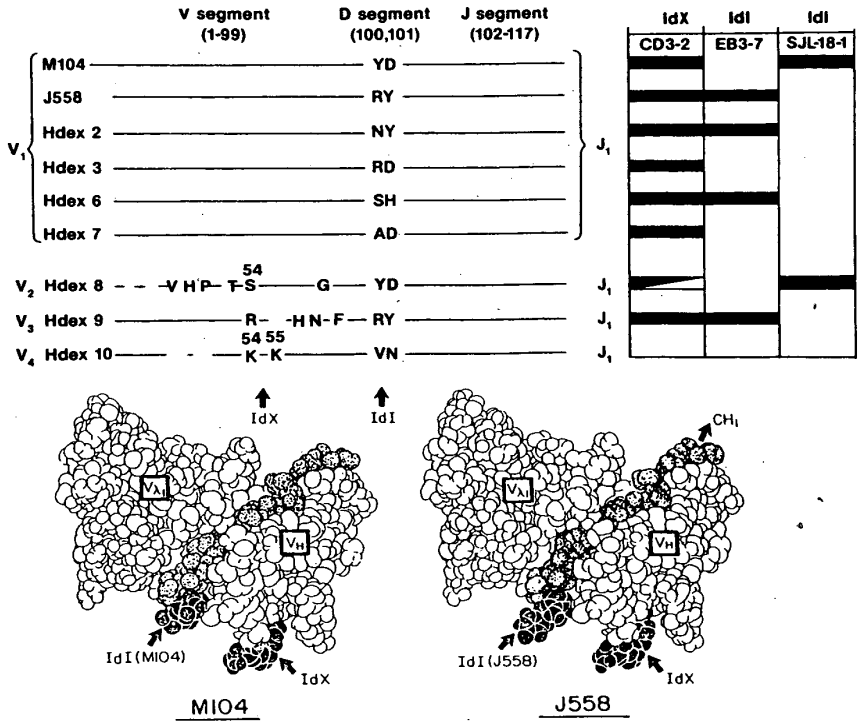


FIG. 1. Binding of mouse monoclonal anti-idiotope antibodies to anti-α1-3 DEX specific antibodies. Reactivity is shown by *black horizontal bars* of three monoclonal anti-idiotype antibodies against a selected panel of mouse antibodies to α1-3 dextran. The **upper portion** shows the V_H amino acid sequences of myeloma (M104 and J558) and hybridoma (HDEX) derived IgMλ sequences on the left. Differences from the M104 sequences are indicated by the one-letter notation for amino acids. The regions encoded by the V, D, and J segment genes are indicated. Reactivity of the anti-Id antibodies is shown by *black horizontal bars* on the right. It can be seen that reactivity of the anti-IdX antibody CD3-2 is correlated with amino acids 54 and 55 (within the second hypervariable region) and reactivity of the anti-Idl idiotopes is associated with determinants in the third hypervariable region (amino acids 100 and 101). The **lower portion** shows a computer-generated space-filling model of the variable domains of M104 and J558. These proteins differ only in D-encoded residues. J_H-encoded amino acids are shaded in *gray*. The binding sites face toward the lower left.

More advanced molecular analyses provided by site-directed mutagenesis have also confirmed previous observations and added more to our understanding of idiotypy. In particular, as has been shown in other systems, some mutations introduced simultaneously have substantially more effects on idiotope expression than when introduced alone (20–23).

The most definitive methodology for the establishment of idiotope association, by x-ray crystallographic analysis of the idiotype–anti-idiotype interaction, has been applied to only one example. A monoclonal Fab anti-Id–anti-hen egg lysozyme complex has been analyzed and it was found that the idiotope involved 13 amino acid contact residues involving hypervariable regions and one framework region. From this structural analysis, the V region of the anti-idiotype (anti-Id) also appeared to react more with the V_L of the anti-lysozyme antibody than the V_H region. In general, this particular Id–anti-Id complex was similar to that seen in complexes analyzed between V regions of antibodies and other protein antigens (24).

Recently, a number of interesting antibody–idiotype interactions have been described and have given rise to new terminology. Certain antibodies, notably the mouse plasmacytoma protein TEPC15, which is specific for phosphorylcholine, have shown the property of binding to themselves. This and other such antibodies have been termed autobodies. The self-binding locus (within the T15 molecule) was shown to be associated with a peptide sequence involving the second hypervariable region extending into the third framework region of the T15 V_H region (25). Another peculiar anti-idiotypic antibody (epibody) was shown by Bona et al. (26) to react with not only the idiotype on a monoclonal human anti-IgG au-

tobody (a rheumatoid factor) but also the target antigen Fc of human IgG. These antibodies are termed epibodies and Carson and his colleagues have provided evidence that the structural basis for such an epitope is a Ser.Ser.Ser sequence shared by the IgM RF light chain and the Fc portion of IgG (27). These two examples exemplify the remarkable heterogeneity of Id–anti-Id interactions; however, the functional significance of such antibodies is not known at this time.

In summary, idiotypes and anti-idiotypic antibodies are extremely useful tools in the functional analysis and delineation of V regions expressed by B cells and in serum antibody responses. They have also been used in studies on inheritance of genetic markers on Ig V genes, mapping of V genes, and many aspects of the regulation of V gene expression (28,29). However, in the normal individual animal, the nature of idiotype and anti-idiotype recognition may be extensive and involves not only idiotopes of B cells but also idiotopes of T cells. Also, the interactions of these cell lineages through idiotypes on T and B cell V regions are physiologically important in the regulation during development and maintenance of the immune system.

NETWORK THEORY

Drawing on this background of idiotypic expression defined in immunoglobulin molecules, Niels Jerne (30), in 1972, first proposed that the immune system was regulated internally via idiotype recognition when he stated: "It therefore seems likely that antibodies arising from antigenic stimulation of a set of lymphocytes suppress other lymphocytes and that the entire system represents a complex interacting network of expression and suppression of potentialities." His ideas were based on the observed extreme heterogeneity of variable region structures of immunoglobulins and he reasoned that each immunoglobulin molecule not only expressed a nominal antigen-binding site for a particular antigen (defined as the paratope) but by virtue of the antigenic determinants expressed on the variable reigns (idiotopes) could also be recognized by other antibody molecules. These latter antibodies were later referred to as Ab2 (or anti-idiotypic antibodies), while the original Ab with a particular paratopic specificity was referred to as Ab1. Another major postulate of this theory was that, because of the apparently enormous potential for Ig V gene diversity, the idiotope repertoire must mimic the large universe of naturally occurring nonself external and self antigen epitopes. Such immunoglobulin-borne determinants or idiotopes were termed the internal images of antigens. It is, however, now clear that the functional distinction between anti-idiotypes and the internal image developed in his original hypothesis is wrong and that symmetry at least in functional terms may be the dominant force in network interactions. This theoretical concept has been exploited, however, in a practical way in attempts to isolate antibodies that mimic particular external antigens (29). Also, it has been used to construct antibodies to molecules such as membrane receptors that may be expressed normally at low levels on membranes and are difficult to isolate by conventional immunization procedures.

Jerne's overall idea was that the immune system was in a state of equilibrium in that Ab2 antibodies held Ab1 production in check and that introduction of antigen for which Ab1 was specific would interrupt these interactions in a mutually interactive way: stimulating Ab1, which in turn suppressed Ab2, permitting the growth and differentiation of Ab1 B cells and secretion of Ab1 molecules. Jerne originally envisioned structures constituting the idiotope and paratope on V regions, which were functionally different. This led to the assumption of asymmetrical interactions between antibodies that expressed Id or anti-Id, so that the interaction between idiotype and paratope was undirectional in its proposed functional activity. As first illustrated by Jerne (31), paratope would suppress the target idiotope and idiotope would stimulate cells bearing the appropriate paratope; for example, idiotope stimulates → paratope and idiotope ← suppresses paratope. Since these initial theories were formulated, network interactions have generally been considered to be symmetrical in that the directionality of a given reaction between Id1 and Id2 can proceed in both ways, Id1 stimulates ↔ Id2 or Id1 suppresses ↔ Id2. In its original form, Jerne's network theory was commonly viewed to not include T cells. However, in both early publications on the subject he clearly had a central role for T cells in the network. In 1972 he wrote: "If T cells can suppress such B cells, the target of this type of suppression would seem to be the antigenic determinants of the receptor molecules of these B cells since these are the only targets that distinguish different B cells. Furthermore, it would seem that these targets are recognized by the combining sites of the T cell receptors" (30,31). Subsequent experimental work has shown that T cells can also be involved in interactions with B cells and their Ig products and these will be discussed later. Also, it is clear that the network theory must be modified due to the subsequent delineation of subsets of T and B cells, which are functionally distinct and alter our concept of network interactions.

Theoretical Models of Idiotypic Networks

The advent of Jerne's idiotypic network theory provided a stimulating challenge to theoretical immunologists and produced a flurry of theoretical analyses of the immune system from a modeling viewpoint. Some of the earliest were supportive of the asymmetry of the initial

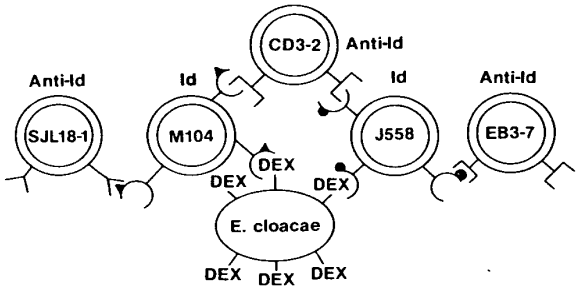
Jerne model and incorporated his ideas into a mathematical model (32). However, it became apparent that the distinction between Id and anti-Id was probably invalid and the asymmetric consideration of such interactions soon gave way to theoretical models based on symmetrical network functions. The symmetrical versus nonsymmetrical function of networks was first described and developed by Hoffman in 1980 (33,34). This idea at first, while theoretical, was an important concept to be accepted, albeit slowly, by experimental immunologists who tended to become fixed on the directionality of Id–anti-Id interactions. These concepts have now been extended to modeling of immune responses to a variety of infections including AIDS (35). Although modeling of network interactions has developed slowly over the last 15 years, specialized theoretical applications such as models applied to the developing immune system (36–38) have led to bridges between the ideas of experimentalists and network theoreticians (39). Although the models produced by network theoreticians have been stimulatory and a challenge to experimental immunologists, they have not yet produced a series of predictions that have helped in directing experimentalists to test these predictions. In fact, up to now most modeling has relied on a number of empirical observations in order to build and expand the various network hypotheses. Some of these empirical observations are discussed in the next section.

Supportive Evidence for the Existence of Idiotype-Directed Interactions and Maintenance of the Immune System

At this point, consideration of several examples of idiotype-directed manipulation of immune responses will not only illustrate the experimental evidence that indirectly supports the concept of idiotypic networks but will also illustrate how idiotypic intervention with appropriate reagents can cause long-lasting effects on the repertoire of B cells involved in particular immune responses.

It has been well established since the original description by Strayer and Köhler (40) that administration of anti-idiotypic antibodies in small quantities, especially in the neonatal period, leads to the severe and chronic suppression of that particular idiotype (41,42). There are many descriptions of idiotype-specific suppression with both heterologous and monoclonal anti-idiotypic antibodies (reviewed in refs. 40–44). However, for continuity, examples within the anti-DEX and anti-PC system are considered here since these have been dealt with in several laboratories and there is a better developed link between what is known of the structure and nature of idiotopes on these antigen-specific Abs and the functional activity of corresponding anti-idiotype antibodies.

Immunization of appropriate strains of mice with the purified polysaccharide α1-3 DEX or in forms associated with bacteria such as *Enterobacter cloacae* will induce a vigorous anti-DEX response consisting largely of antibodies bearing the J558 idiotype. If neonatal mice are given a small amount of any of three anti-idiotype antibodies specific for M104E or J558 idiotopes (Fig. 1) and the mice are permitted to grow until 6 to 8 weeks of age before challenge with α1-3 DEX, antibodies expressing the relevant idiotypes are almost totally suppressed. EB3-7 suppresses the J558 idiotope but does not affect M104E Id-bearing molecules detected by the monoclonal antibody SJL18-1 (see Fig. 2). Thus neonatal treatment with anti-idiotypic antibodies can lead to a permanent loss of clones of B cells expressing the particular idiotype. The extent of the loss depends on the amount of cross-reactivity of such anti-idiotypic antibodies. Neonatal treatment with antibody CD3-2, which, as seen in Fig. 1, reacts in an idiotype-specific fashion with the majority of the clones of anti-DEX antibodies (as represented by the anti-DEX series of hybridomas and plasmacytomas), therefore produces a much stronger overall suppression of the total anti-DEX response than when the Id is suppressed because a proportionally smaller number of potential anti-DEX clones are affected by the



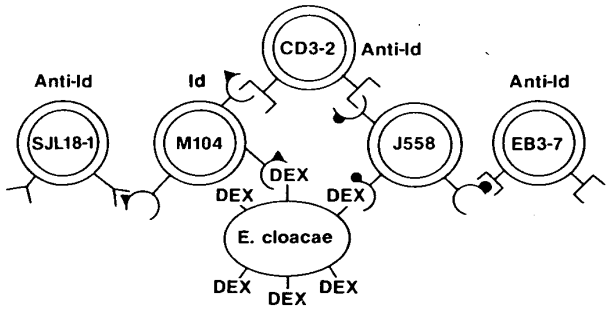
Anti-Id Treatment	Serum Antibody λ-Anti-DEX	J558+	M104+	CD3-2
Saline	383.9	226.8	90.8	High
EB3-7	241.7	<0.5	122.7	High
SJL18-1	344.8	54.0	<0.5	High
CD3-2	75.6	0.8	<0.5	Absent

FIG. 2. Idiotype levels in mice neonatally suppressed with monoclonal anti-idiotype antibody. Diagram represents the potential interactions of the anti-Idl antibodies SJL18-1 and EB3-7 with the M104 and J558 idiotypes, respectively, with the IdX anti-idiotype CD3-2 reacting with both idiotypes. Administration of these antibodies to mice at 1 day of age followed by challenge with α1-3 dextran or the bacterium *Enterobacter cloacae* when they were adults showed the typical effects of such neonatal treatment. Anti-Idl antibodies suppressed only a portion of the anti-DEX response while the anti-IdX (CD3-2) suppressed the overall response much more strongly.

anti-Id1 antibodies when adult mice are stimulated with DEX (14).

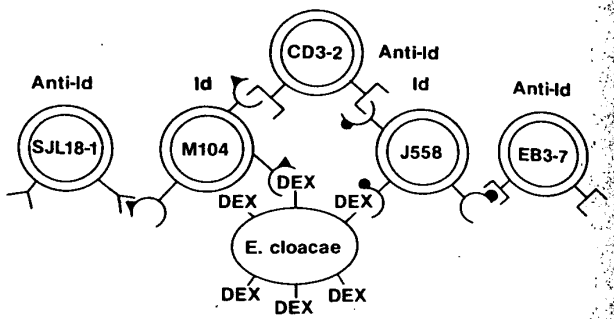
Such effects on other idiotypes can be transmitted from the maternal blood circulation (14). As an example, in the same experimental system described in Fig. 2, delivery of the anti-J558 anti-Id antibody *in utero* by passive maternal transfer following administration to pregnant mice leads to a long-lasting suppression of the J558 Id in offspring as seen in Fig. 3. It is of note that *in utero* transmission of IgM antibodies does not occur in mice, so that IgM anti-Id antibodies are not suppressive via this route. Techniques, such as a T-cell-independent splenic focus assay, that detect functional B cell precursors fail to demonstrate the presence of B cell precursors specific for α1-3 DEX that express the J558 idiotope (Fig. 4). These results suggest that there has been a functional deletion of these B cells, although other mechanisms may be operating (*vide infra*). Moreover, these results are consistent with the frequently reported susceptibility of B cells, during early development, to functional inactivation by crosslinking of their sIgM antigen receptors by anti-μ antibodies or antigen (45,46). In this case crosslinking of Ig receptors via idiotypic determinants appears to functionally delete the B cell precursors (14).

The maternally transmitted effects of such antibody administration have been demonstrated in other systems. For example, appropriate immunization of pregnant mice with an idiotype leads to maternal production



	Route	Lambda-Positive Ig (μg/ml) anti-DEX	EB3-7+	SJL18-1+
EB3-7	in utero	318.2	< 0.5	48.3
EB3-7	post-partum	794.0	< 0.5	157.3
Control	post- partum	478.5	137.4	82.0

FIG. 3. DEX-specific antibody levels after maternal treatment with MAIDs. In the same model described in Fig. 2, both *in utero* and postpartum administration of the IgG1 anti-Id1 antibody EB3-7 resulted in transmission of these antibodies to the developing fetus or neonate and induced a suppression of anti-DEX antibodies expressing the J558 idiotype following immunization. Total anti-DEX and the associated SJL18-1 idiotype are, however, comparatively normal.



	% of anti-DEX precursors EB3-7+	SJL18-1+	CD3-2+
Control	74	26	83
EB3-7-suppressed	0	50	63

FIG. 4. Functional deletion of idiotype expression among DEX-specific B cell precursors. In the same model system described in Figs. 2 and 3, the frequencies of B cell precursors expressing EB3-7- and SJL28-1-defined idiotopes were determined in the splenic focus assay. As shown, mice that had been treated at birth with EB3-7 as adults completely lack functional precursor B cells. M104 idiotype-bearing anti-DEX precursors are, however, doubled in frequency, suggesting that the M104 B cell precursors have compensated for the loss of J558 B cell precursors.

of a syngeneic anti-idiotype response. Maternal transmission of these anti-idiotypic antibodies results in suppression of that idiotype in the offspring (47,48). Thus the maternal effects on the final B cell repertoire expressed by her progeny may be substantial, although little is known regarding the consequences and possible induction of naturally occurring anti-idiotype antibodies. It must be stressed that many of these maternally induced effects result from nonphysiological immunological manipulations of the mother, and whether maternal–fetal effects influence neonatal B cells under normal conditions is not clear. Similar effects, although not as yet so widely investigated as those involving manipulation of the B cell repertoire, can also occur with developing T cells. Neonatal administration of anticlonotype antibodies such as KJ16 will delete a subset of brightly staining T cells in the peripheral lymphoid tissues of the animals, although dimly staining "immature" cells can be found in the thymus (49). Presumably, other examples of anti-idiotypic or clonotypic manipulation of the developing T cell repertoire may easily be accomplished with the appropriate reagents.

Idiotype Connectance

Some examples of the specificity of anti-idiotypic manipulations and the long-lasting effects of such manipulations have been described above. However, the reper-

toire of B cells is said to be complete because of the considerable diversity of B cell V regions generated by the genetic mechanisms described in Chapter 10. Completeness, in the simple sense, means that all immunoglobulin molecules or receptors can potentially react with one or many other complementary idiotype-bearing structures. Because of the extent of this diversity, it is probable that idiotypes can be shared between different antibodies involved in response to disparate antigenic determinants (50–52). Thus it is proposed that certain idiotypes are determined possibly by framework or CDR regions that are shared by, and form links between, otherwise structurally disparate V regions (*vide infra*). Such idiotopes have been designated regulatory idiotopes since they would permit a more limited number of idiotypically determined interactions to be included in any proposed regulatory circuits involving either complementary B cells and/or antibodies, and T cell receptors that react with B-cell-derived antibody V regions (52–54). Although there is little substantial evidence to support such an idea, the recent demonstration of shared V region sequences on topographically solvent-exposed parts of human Ig V_H regions, which are shared between certain distinct V_H families and also show remarkable conservation with similar related V_H framework regions in mice, would provide a structural basis for regulatory idiotopes on Ig molecules even of different specificities (55).

Perhaps the greatest apparent degree of idiotypic interactions or connectance occurs early during B cell development. This has been demonstrated by constructing panels of hybridomas from mouse fetal or perinatal liver, which is the site of B cell generation early in development, and from neonatal spleen. Analysis of the IgM antibodies produced by these hybridomas has shown several striking characteristics that differ from that of similar panels of hybridomas derived from nonimmunized adult mice. First, these antibodies are frequently multispecific, in that they react in solid-phase binding assays with more than one antigen (56–58). Although most of such studies have been performed in mice, analysis of IgM immunoglobulins from Epstein–Barr virus- (EBV-) transformed human cord blood IgM B cells shows a similar pattern of reactivity (59). These IgM antibodies in both mouse and human are particularly reactive with self-antigens such as intermediate filaments and other intracellular or sequestered antigens. However, of greater interest, particularly in mice, is that many of these antibodies show autoreactivity to well-characterized syngeneic immunoglobulin idiotypes (56–58).

More recently, sequence analysis of fetal and neonatal hybridoma Ig V genes or those derived by PCR analysis has shown that the early B cell repertoire differs considerably from the adult in the expression of V gene segments. It has been shown that heavy chain V gene segments do not express N segments (60). Furthermore, there is a preferred use of reading frame in D-J joinings. As described in Chapter 10, there is also a preferred use of certain D and J segments (61,62). These factors, coupled with the lack of somatic hypermutation, promote a relative restriction in the early fetal compared to the bone marrow-derived B cell repertoire in the adult. The genetic mechanisms at work in the production of differentiating B cell fetal repertoire versus the adult also provide an opportunity for more sharing of V region idiotopes between otherwise different B cell receptors and probably account for the connectivity seen in the early repertoire.

From other studies it has also been shown that, during ontogeny in a variety of species, there appears to be a hierarchy in the development of the ability to make an antibody response to different antigens (see Chapter 12). A summary of some of these findings in mice is described in Fig. 5. As can be seen, the earliest B cell precursors that can be detected in mice frequently have DNP or other hapten-specific binding capabilities (and

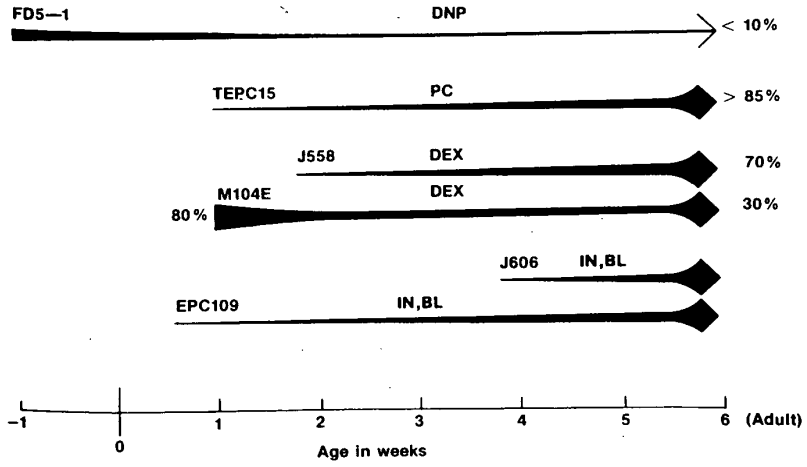


FIG. 5. Diagram depicts the emergence during B cell development of the ability to make antibody responses to a variety of bacterial associated antigens and haptens. On the left side of each developmental arrow is the idiotope preferentially associated with a particular response. Percentages when present indicate the frequency of antigen-specific B cells expressing a particular idiotope at that time in development. (Data summarized from refs. 64–66, 70, and 71.)

frequently express an idiotope defined with an anti-idiotypic antibody such as FD5-1) (63–65). Other specificities characteristically appear later. For example, the T15 idiotype response to phosphorylcholine does not appear until day 4 after birth (66–69), while the response to α1-3 dextran occurs on day 10 (70) and the responses to inulin and α1-6 dextran occur even later (29,47,48,71,72).

These results are summarized from studies in mice. However, there is clear evidence that in humans there is a delay in the ability to respond to certain antigens and, certainly, there is a delay in the onset of the ability of infants to respond to polysaccharide antigens (73–76). A particularly well-studied example of the restricted heterogeneity and idiotype flux that occur during the human perinatal period is found in the responses to the capsular polysaccharide of *Haemophilus influenzae* (HibPS) (76). This response is of limited heterogeneity (77) and molecular and idiotypic analyses of this response during development show that there are dramatic changes in the V region usage of anti-HibPS antibodies (77–79). In this respect this restricted human antibody response is similar to the examples of idiotype profile changes to these polysaccharide antigens observed in mice. Although there are other reasons proposed for this hierarchy, such as delayed maturation of functional auxiliary cells, there is also evidence that these differential rates of appearance of functional precursors are the result of a programmed development of the B cell repertoire and that this depends on interactions between B cells and/or their immunoglobulin products as outlined later.

Idiotype-Directed Interactions and Their Role in Development of the B Cell Repertoire

It has previously been reported that in responses to certain antigens there is a flux of idiotypic and anti-idiotypic antibodies and that a wave of anti-idiotype-producing B cells in spleen may precede the later appearing antigen-specific B cells (80). Similarly, during development of the T15 idiotypic response to phosphorylcholine, it has been shown that anti-idiotypic antibody-secreting B cells precede or coincide with the development of T15 Id-expressing phosphorylcholine-specific B cells (81,82) and, as shown in Fig. 6, direct examination of B cells during the perinatal period indicated that anti-T15-bearing B cells preceded the appearance of target T15 idiotype-bearing cells (67).

Appropriately selected idiotypic and anti-idiotypic antibodies, in particular those selected from the neonatal repertoire established by the collection of IgM-producing hybridoma isolated by Holmberg et al. (56), provide evidence that there exists in the serum of adult mice a reciprocal relationship between such Id and anti-Id. Injection of neonatal animals with very small amounts of either Id or anti-Id antibodies produces a

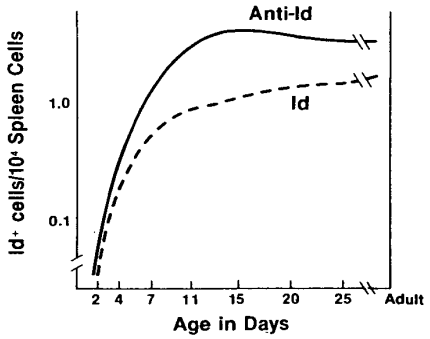


FIG. 6. Description of frequency of T15 idiotype and anti-T15 Id-bearing B cells during ontogeny. B cells were directly detected and enumerated by fluorescence microscopy. As can be seen, anti-Id B cells preceded the development of Id-bearing cells. For further details, see ref. 67.

long-lasting alteration in the serum Id–anti-Id profiles so that these antibodies, or the B cells from which they were derived, appear to be maintained in an equilibrium that depends on the relative concentrations of the Id–anti-Id pair (83).

In similar studies, the role of the early appearing multispecific B cells, described in the previous section, was studied in the neonatal period with respect to their effects on emerging B cells and their contributions to the overall diversity of the adult B cell repertoire. As shown in Fig. 7, it was possible to construct a mini-network by appropriate *in vitro* analyses that describes the reactivities of a set of neonatally derived antibodies that connect the anti-PC and anti-DEX responses. To determine the physiological relevance of such interactions, various manipulations designed to test this apparent connectivity were carried out in neonatal and adult mice.

To determine whether deletion of the early appearing cells affects the cascade of proposed B cell interactions depicted in this diagram, mice were treated with an anti-idiotypic antibody FD5-1 (Ab4) by the maternal route in an attempt to delete DB3-like cells *in utero* or were treated at various times after birth. When these animals were tested as adults for anti-PC and anti-DEX response as shown connected in Fig. 7, it was found that early perinatal administration abrogated the anti-PC response while leaving intact the anti-DEX response. In contrast, treatment at days 10 to 15 postpartum markedly affected the anti-DEX response but left the anti-PC response intact (Fig. 8) (42).

The second kind of related experiment was to determine whether administration of an antibody such as BD2 at appropriate intervals could modulate the responses to PC and DEX as would be predicted by the model network in Fig. 7. As can be seen (Fig. 7), BD2 reacts with T15 and J558 idiotypes, which are two structurally distinct molecules, that possibly express a common regulatory idiotope. Administration of BD2 en-

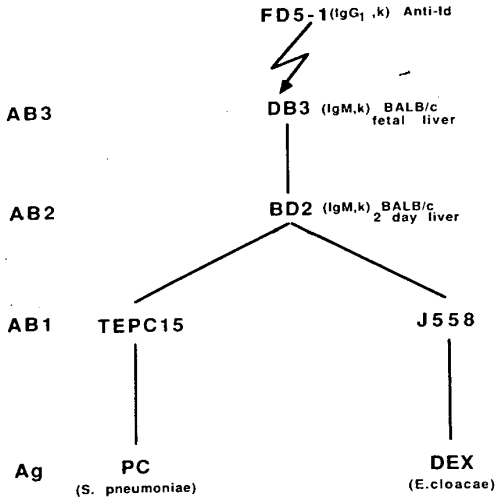


FIG. 7. A simplified diagram of idiotypically connected antibodies derived from hybridomas constructed from perinatally derived B cells. Antibodies DB3 and BD2 are IgM antibodies derived at the periods of development indicated. FD5-1 is a $\gamma 1,\kappa$ hybridoma anti-idiotype antibody against M460 (a BALB/c DNP-binding antibody). This particular anti-idiotype antibody reacts with a large number of the multispecific fetal liver-derived hybridomas and was used to idiotypically suppress such cells *in utero* (via placental transfer) or by direct administration after birth as described in Fig. 8 (see also ref. 42).

hanced the response to PC and DEX, respectively, in an idiotype-specific manner (Fig. 9) (42).

In both of these experiments there appear to be developmental windows, early for the anti-PC and later for the anti-DEX responses, when the suppressive effects of FD5 and enhancing effects of BD2 were most effective (Figs. 8 and 9). These time points correlate with the periods in normal animals when, as shown in earlier experiments and in the top panels of Figs. 8 and 9, precursor B cells for these responses appear and begin to expand rapidly in number. These kinds of experiments and others suggest that these early multispecific, idiotypically reactive B cells operate in a cascade-like fashion in order to facilitate the differentiation and/or proliferation of the later appearing B cells in an idiotype-specific manner. In contrast, manipulations with these same antibodies later in the life of the animal appear to have little effect on the already formed adult B cell repertoire. The concept that events that occur early in development play a major role in the subsequent development and shaping of the specificity of the B cell repertoire also appears to hold true for certain T cell–B cell idiotypic interactions and is discussed later. The overall conclusions from these kinds of experiments are that B cell interactions mediated through idiotypes occur early in development and have a long-lasting effect on the diversity of the B cell repertoire that is established in the adult.

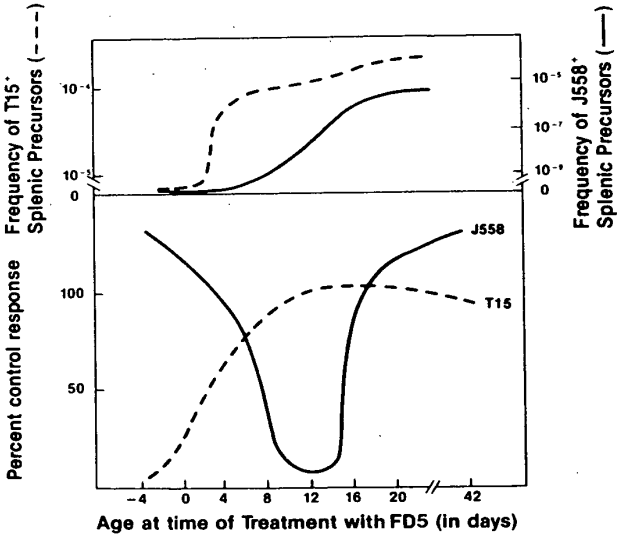


FIG. 8. The effects of perinatal administration of FD5-1 on the idiotypically linked antibody responses to PC and DEX (**B**) compared to the normal development sequence of B cell precursors with specificity for PC and DEX and expressing the T15 and J558 idiotypes, respectively (**A**).

Clonal Dominance

In inbred strains of mice, the antibody responses to certain antigens have been shown repeatedly to be relatively restricted in heterogeneity. These include the responses to phosphorylcholine (T15Id) (84,85), α1-3 dextran (J558Id) (86,87), ARS (CRI_{ARS}) (88), and NP (the early anti-NP antibodies, NP^bId) (89). Structural studies of such antibodies have shown that they express identi-

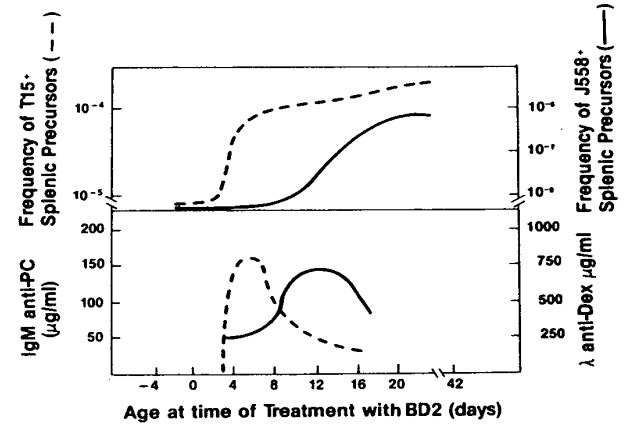


FIG. 9. The effects of postnatal administration of the IgM, anti-idiotype antibody BD2 on the development of antibody responses to PC and DEX (**B**) compared to the normal developmental sequence of B cell precursors with specificity for PC and DEX and expressing the T15 and J558 idiotypes, respectively (**A**).

cal, or very closely related, heavy and light chain V regions. In many cases, these dominant antibodies do not appear to have any obvious selective advantages, such as a greater affinity for antigen, which would contribute to their expansion and subsequent dominance. In some cases, it has been shown that these idiotypes come to dominate the preimmune repertoire prior to exposure to externally derived antigen and in the absence of T cells. This suggests that selective forces based on antigen affinity and T-cell-directed effects may not control the expressed preimmune Id dominance (90). Based on the work of Köhler and Fung (91) and the observations described in the previous sections, a model has been developed for the mechanism of establishment of clonal dominance in one of the homogeneous antibody responses typified by the T15 dominant anti-phosphorylcholine response (Fig. 10). In this model, the central role for establishing Id dominance is attributed to the early development of a set of anti-idiotypic cells that expand the $T15^+$ precursors at the expense of other non-T15 precursors. The result is that B cells bearing this idiotype come to dominate the preimmune repertoire prior to antigen exposure.

In recent experiments, it has been shown that administration of antigen in the form of a vaccine during the perinatal period of development, when these proposed interactions are occurring, results in dramatic changes in the repertoire of adult mice. For example, administration of *Streptococcus pneumoniae* vaccine changes the profile of the adult mouse so that the normally dominant T15 idiotype is no longer expressed and protective antibody is no longer produced. Furthermore, the idiotypically connected anti-DEX response was essentially ablated (92,93). These experiments again support the idea that the early developing repertoire is characterized by a carefully regulated set of interactions, which upon manipulation results in long-lived imprinting of the repertoire.

That this establishment of dominance occurs only during the critical perinatal period is also suggested by experiments in which bone marrow or fetal liver is transplanted into lethally irradiated mice. Under these conditions, in both the anti-PC or anti-ARS system, the normally dominant idiotypes do not arise. However, it has been shown that administration of small amounts of anti-Id to T15 or CRI_{ARS}, at the time of transfer, would reconstitute the dominance of these two particular idiotypes following immunization with PC or ARS (94–96). These experiments reiterate that the normal development of the B cell repertoire is influenced by early ontogenetic events and that the effects of these early interactions appear to be long lasting and may not be reproduced during adult B lymphopoiesis.

Silent Idiotypes

In the response to certain antigens in which a particular idiotype dominates, there may exist other antigen-specific B cell precursors from which antibody is not secreted or which constitute a minority of the responding cells. However, after administration of appropriate anti-idiotype or idiotype, these particular precursors can be expanded to make a substantial contribution in the antibody response to that antigen. In a well-studied example, the ability to respond to two different epitopes on the same fructosan molecule was shown to occur at widely different intervals after birth. One-day-old mice can produce a humoral immune response to the β2-6 fructosan epitope, whereas the ability to respond to the β2-1 epitope does not appear until 1 month when the characteristic idiotype, A48Id, is expressed. However, treatment of neonatal mice with antibody expressing the A48Id permits the expression and early detection of that particular idiotype in antibody responses to fructosan (97,98). These results have been proposed to be due to expansion

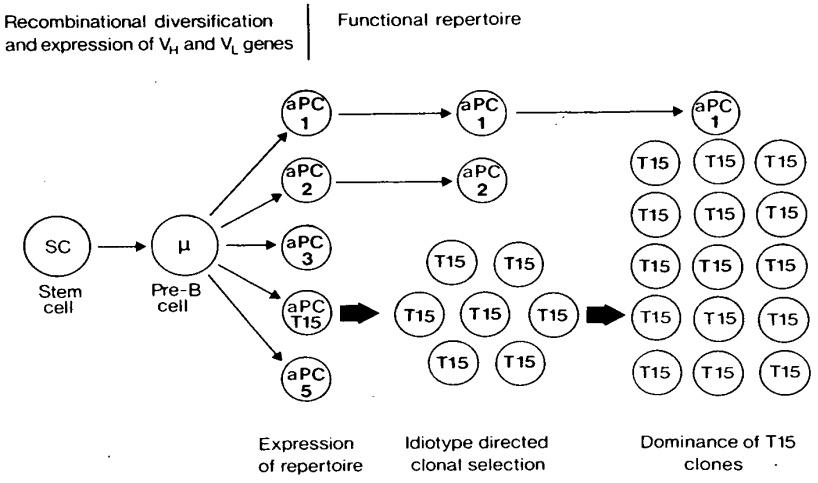


FIG. 10. A model for the neonatal establishment of clonal dominance of the T15 idiotype in response to phosphorylcholine in the repertoire of adult BALB/c mice.

of an A48 idiotype-specific set of helper T cells, which then expand this idiotype. However, it is not clear in these experiments what the effect of the introduction of A48Id-bearing Ig is on reciprocal sets of B cells.

Interactions Between T and B Cells Through Idiotypes

As outlined in Chapter 4, the primary selection of the T cell repertoire occurs within the thymus. It is here that the interactions of the developing thymocytes with major histocompatibility (MHC) products, expressed on certain specialized MHC-expressing accessory cells, result in the characteristic MHC-restricted nature of self/nonself discrimination typical of immunocompetent T cells. However, there have been frequent reports in the literature of idiotype-specific T cells and there is also evidence for the subsequent education or selection of peripheral T cells through idiotype-related interactions with B cells and/or their immunoglobulin products (99–112).

This is a controversial area, since the varieties of idiotype-reactive T cells examined appear to depend on the laboratories in which these experiments are performed. There are examples of idiotype-specific T cells that are characteristically MHC restricted and presumably recognize the Ig idiotype in the form of a peptide in association with class II antigens (107,109–111). One of the best characterizations of these is recognition of an idiotype on a myeloma protein MOPC315. These residues include 91 to 108 of the λ2 light chain and have been shown to be processed in cells that synthesize this particular IgA protein and are presented as a peptide on class II antigens (111). On the other hand, idiotype-specific T cells can be depleted or activated by treatment with immunoglobulin in the apparent absence of MHC-associated presentation. This points to the possibility of two kinds of T cell–Ig idiotype interactions: one being a direct involvement of Ig idiotype with T cell receptor that is not MHC restricted, the other being MHC restricted (104,106).

An example of developmental interactions of this kind is that involving an idiotype on T cells detected by a monoclonal antibody, F6(51), originally made against a DNP-specific plasmacytoma protein M460. F6(51) was one of many anti-Ids tested that reacts with a population of helper T cells induced by DNP coupled to syngeneic cells. This population of T cells was shown to express the F6(51)-defined idiotype on a T cell receptor with specificity for DNP. Thus this antibody will precipitate the characteristic T cell $\alpha\beta$ heterodimer and will inhibit both proliferation and interleukin 2 (IL-2) and IL-4 production by these cells in response to exposure to DNP-coupled syngeneic spleen cells. The anti-Id will also inhibit helper T cell activity, preventing proliferation and differentiation of DNP-specific B cells (108). In this model it was shown that either immunoglobulin or B cells expressing Ig were necessary to select this population of Id^+ T cells. If anti-μ administration is begun at birth and continued for 3 to 4 weeks after birth, suppression of the normal development of B cells occurs. Upon cessation of anti-μ administration, a normal B lineage with respect to numbers of cells, Ig serum levels, and lipopolysaccharide (LPS) responsiveness is rapidly established. In such cases, however, the mice do not develop the population of helper T cells expressing the F6(51) Th clonotype. Another experiment that produces a similar deficit in the F6(51) Th population results after x-irradiation and BM reconstitution of adult mice. In this case, the F6(51) Th population also fails to develop. These results show that the development of the F6(51) Th population requires interaction with an idiotype-bearing immunoglobulin. Such interactions must occur within the first 3 weeks of life and, if interrupted, cannot be regained. The selection of F6(51) Th cells can also be affected by maternal transfer of these anti-receptor antibodies. If pregnant mice are immunized with M460 and induced to produce F6(51)-like anti-Id IgG antibodies, these can cross the placenta and suppress the development of F6(51) Th cells for the life of the offspring. Likewise, the administration of this IgG anti-Id antibody by neonatal injection will also suppress development of F6(51) Th cells. These observations parallel those described earlier in this chapter, in which it is proposed that B cell interactions between immunoglobulin idiotypes result in the dominance of certain clonotypes. Such selective processes appear to be restricted to certain windows of time during development, a period during which both T and B cell repertoires are extremely malleable and susceptible to external manipulation.

Thus in the development of both the T cell and B cell repertoires, there appears to be a crucial period, early in ontogeny, when interactions between the T cell and B cell populations result in long-lasting effects on the adult repertoire. Later on, the two apparent kinds of idiotype-recognizing T cells have helper activities as outlined in the above discussion or manifest suppressor activity on B cell function. How then do these interactions occur? In Chapter 17 the basic concept of T cell recognition by the interaction of the T cell receptor with peptides associated with MHC products is discussed. An example that shows this type of recognition clearly, with respect to immunoglobulin, is the peptide associated with a λ2 light chain from a mouse plasmacytoma protein that could induce a population of helper T cell clones to proliferate. These T cells recognize the peptide in the context of a MHC product rather than the whole Ig molecule (111). However, as discussed earlier, it is probable that an antibody may react directly with the T cell receptor by virtue of its three-dimensional structure. This would imply that certain subsets of B cells may interact directly with T cells, leading to their non-MHC restricted expansion. This pro-

cess may then be responsible for the development of T cell repertoires that are no longer self-MHC restricted and lead to aggressive pathological conditions and autoimmune diseases (105).

As mentioned earlier, there is some evidence that the CD5 (B1) B cells may be involved in this selection procedure. It is of particular interest that certain IgM hybridoma antibodies derived from the panel of neonatal hybridomas obtained by Holmberg et al. (56) can mimic the activities of the F6(51) anti-idiotype antibody. Thus these early IgM antibodies may play a role in directing the normal development of F6(51) Th cells during the critical neonatal period of repertoire development. In addition, D. Holmberg et al. (*personal communication*) have shown that introduction of purified antibodies of this kind into newborn NOD (nonobese diabetic) mice will prevent the diabetes from producing T cell-mediated insulitis that develops in adult mice of this strain, so it is clear from these kinds of observations that there is an interplay between Ig and T cell receptors. However, molecular evidence to strengthen these observations, except for the work already described, is not as yet abundant.

Idiotypic Vaccines

As proposed by Niels Jerne in his original model, idiotopes on lymphoid receptors may in some cases mimic external antigens because of the extensive diversity of the immune system. This idea has prompted many attempts to use the internal image of a foreign antigen, mimicked by the idiotypes of T or B cell receptors, to act as targets for anti-idiotypic antibodies (106). In this way, it is proposed that anti-idiotypic antibodies may induce populations of T or B cells that can bind the extrinsic antigen. While these findings have been useful in studying the interactions within idiotypic cascades following the introduction of a foreign antigen, widespread efforts have been made to use such anti-idiotypic antibodies as vaccines. Many of these studies are summarized in a recent review (107). Why use such an approach when modern molecular and recombinant DNA technology should be able to provide large amounts of genetically engineered antigens, which would be useful in constructing suitable protein vaccines? There are cases where an anti-idiotypic mode of induction of a response may be useful. If a given epitope of a protein is discontinuous and results from three-dimensional folding, then an anti-Id could be made that would mimic that structure. The ability to respond to polysaccharides is delayed in both the human and mouse, and it has been shown that useful responses in mice to *Escherichia coli* capsular polysaccharide could be induced by the administration of anti-idiotypic antisera to suitable anti-polysaccharide antibodies (113, 114). Similarly, T15 antibody responses could be boosted considerably by administration of anti-T15 antibodies (115). However, as outlined earlier in this chapter, it was shown that during development, normally silent clones could be induced in the repertoire at an earlier stage of development by the administration of such antibodies and result in an earlier protective immune response (97,98).

Additionally, in the case of immunization against latent and/or immunosuppressive viruses, there is the possibility of deleterious effects and the use of attenuated viruses and disease caused by measles, mumps, and rubella is sometimes not desirable for these reasons. In this case the production of an anti-idiotype antibody that would induce protective antibodies to the virus would be particularly useful, as well as in immunity to the recently described (HTLV-1, -II, -III) human retroviruses.

In the laboratory setting have there been successful demonstrations of the use of anti-idiotypic vaccines? An anti-idiotype antibody made against a given B cell Ig receptor could react with the receptor and, by crosslinking the targeted B cell, could lead to the activation and clonal expansion of the targeted B cell. However, it is clear that, in order to get an effective antibody response and the induction of a memory response, T cell help is necessary. In this respect it seems that the use of heterologous anti-idiotypic antibodies, for example, by the introduction of rabbit anti-idiotypic antibodies into mice, appears to provide a greater opportunity to induce the production of the appropriate idiotype and the desired specific antibody production. In most cases reported, the amount of specific antibody produced is less than the total isotype-bearing immunoglobulin induced. This is probably due to the Oudin–Cazenave phenomenon described earlier in this chapter. It is likely that the foreign epitopes on the heterologous antibody provide carrier determinants for helper T cells to induce stronger antibody responses. However, this is not always the case, as anti-idiotype antibodies can induce T cell-mediated immunity in mice to a variety of viruses (summarized in refs. 107, 114, and 116).

As already discussed in Chapter 20, a T cell receptor of a given specificity could react with an anti-idiotypic (clonotypic) antibody and could be triggered to expand, as has been shown *in vitro* for various mitogenic clonotype-specific antibodies. Alternatively, T cells may recognize a processed form of a peptide derived from an anti-idiotype antibody. This may be sufficient to induce a population of idiotype-specific helper T cells to promote differentiation and antibody secretion of the appropriate specificity for a given pathogenic organism. However, there is still a lot to be learned about the mechanism of induction of specific immune responses in relation to the mechanism of action, the use of adjuvants, and the selection of one or more appropriate anti-idiotype antibodies

to produce the maximal response against the intended antigen, the anti-idiotype antibodies being used as surrogate antigens (see Chapter 8).

Idiotypes to Study Receptor–Ligand Interactions

The mimicking of the topography of biological structures by the internal images provided by the variability of immunoglobulin molecules (reviewed in refs. 95, 107, 119, and 120) has provided a potentially useful way to study receptor–ligand interactions. In principle, this involves the production of an antibody (monoclonal or heteroantibody) to the ligand for a given receptor. Some of these antibodies will bear topographical structures that resemble the receptor for the natural ligand. When anti-idiotype antibodies are made against this Ab1, some of the antibodies may bind to the receptor for that particular ligand. There is a particularly well-characterized example of antigen mimicry provided by a monoclonal anti-idiotype antibody to an antibody specific for the reovirus III hemagglutinin. This antibody has also been shown to react with a mammalian β-adrenergic receptor that is a target for this hemagglutinin. Sequencing of Ig V regions and peptide analysis defined epitopes that were associated with ligand–receptor interaction. These studies recently were extended to permit the design of an organic molecule that would interfere with virus–receptor binding (117,118). Such antibodies have been useful in the isolation and characterization of the receptors for several peptide hormones and provide a useful approach when the low-level expression of a membrane receptor makes isolation of sufficient quantities to use as an immunogen impossible or difficult (117,118). Furthermore, such antibodies may act as agonists or antagonists for the particular physiological function of the natural ligand. Anti-idiotype antibodies have been made in this way specific for a number of membrane-bound receptors. Recently, Davis et al. (121), in an extensive analysis of 225 mouse anti-human CD4 antibodies, found only one that had a specificity similar to gp120 for CD4. These authors found the frequency of antibody mimicking gp120 to be very low. The authors point out in this article that the affinity of such interactions may be too low to permit techniques used for isolation of genes (86). However, such approaches have certainly provided biological tools for further studies as exemplified by another use of anti-Ids to recognize a mammalian endoplasmic reticulum (ER) retention signal (122).

CONCLUSIONS

While the physiological significance of the network hypothesis in immune regulation has been questioned, there is an abundance of experimental evidence that idiotype-directed interactions among T and B cell receptors occur widely during certain stages in the normal development and maintenance of T and B cell repertoires, as well as in various disease states. Unraveling some of the complexities of these interactions has been aided by the ability to clone the cellular elements involved. However, often these approaches involved either the nonphysiological intervention with a variety of reagents or removal of such interacting T and B cells from their normal microenvironment. What is seen in these situations with respect to the biology of proposed idiotype interactions may not reflect activities *in vivo*, where interactions of much greater complexity may occur. These interactions may be the direct result of the most complex, diversified, and mobile cellular systems of the body encountering the tremendous diversity of antigen receptors expressed by lymphoid cells.

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CHAPTER 8

Immunogenicity and Antigen Structure

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THE NATURE OF ANTIGENIC DETERMINANTS RECOGNIZED BY ANTIBODIES

Haptens

In the antigen–antibody binding reaction, the antibody-binding site is often unable to accommodate the entire antigen. The part of the antigen that is the target of antibody binding is called an antigenic determinant, and there may be one or more antigenic determinants per molecule. To study antibody specificity, we need to have antibodies against single antigenic determinants. Small functional groups that correspond to a single antigenic determinant are called haptens. For example, these may be organic compounds, such as trinitrophenyl (TNP) or benzene arsonate, a mono- or oligosaccharide such as glucose or lactose, or an oligopeptide such as pentalysine. Although these haptens can bind to antibody, immunization with them usually will not provoke an antibody response (for exceptions, see ref. 1). Immunogenicity often can be achieved by covalently attaching haptens to a larger molecule, called the carrier. The carrier is immunogenic in its own right, and immunization with the hapten–carrier conjugate elicits an antibody response to both hapten and carrier. However, the antibodies specific for hapten can be studied by equilibrium dialysis using pure hapten (without carrier) or by immunoprecipitation using hapten coupled to a different (and non-cross-reacting) carrier, or by inhibition of precipitation with free hapten.

This technique was pioneered by Landsteiner (2) and helped to elucidate the exquisite specificity of antibodies for antigenic determinants. For instance, the relative binding affinity of antibodies prepared against succinic acid–serum protein conjugates shows marked specificity for the maleic acid analog, which is in the cis configuration, as compared to the fumaric acid (trans) form (3). Presumably, the immunogenic form of succinic acid corresponds to the cis form (3). This ability of antibodies to distinguish cis from trans configurations was reemphasized in later studies measuring relative affinities of antibodies to maleic and fumaric acid conjugates (4) (Table 1A). Table 1B shows the specificity of antibodies prepared against *p*-azobenzene arsonate coupled to bovine gamma globulin (5). Since the hapten is coupled through the *p*-azo group to aromatic amino acids of the carrier, haptens containing bulky substitutions in the para position would most resemble the immunizing antigen. In fact, *p*-methyl-substituted benzene arsonate has a higher

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TABLE 1. *Exquisite specificity of anti-hapten antibodies*

Hapten	Structure	K_{rel} of antibody specific for	
A.		Maleic (cis)	Fumaric (trans)
Maleanilate		1.0	<0.01
Fumaranilate		<0.01	1.0
B.		Para-substituted benzene arsonate	
Benzene arsonate		1.0	
o-Methyl benzene arsonate		0.2	
m-Methyl benzene arsonate		0.8	
p-Methyl benzene arsonate		1.9	
C.		Lactose	
Lactose	β gal (1 → 4) glu	1.00	
Cellobiose	β glu (1 → 4) glu	0.0025	

Part A from ref. 4; part B from ref. 5; and part C from ref. 6, with permission.

binding affinity than unsubstituted benzene arsonate. However, methyl substitution elsewhere in the benzene ring reduces affinity, presumably due to interference with the way hapten fits into the antibody-binding site. Thus methyl substitutions can have positive or negative effects on binding energy, depending on where the substitution occurs. Table 1C shows the specificity of anti-lactose antibodies for lactose versus cellobiose (6). These disaccharides differ only by the orientation of the hydroxyl attached to C_4 of the first sugar either above or below the hexose ring. The three examples in this table, as well as many others (1), show the marked specificity of antibodies for *cis–trans*, ortho–meta–para, and stereoisomeric forms of the antigenic determinant.

Comparative binding studies of haptens have been able to demonstrate antibody specificity despite the marked heterogeneity of antibodies. Unlike the antibodies against a multideterminant antigen, the population of antibodies specific for a single hapten determinant is a relatively restricted population, due to the shared structural constraints necessary for hapten to fit within the antibody-combining site. However, the specificity of an antiserum depends on the collective specificities of the entire population of antibodies, which are determined by the structures of the various antibody-binding sites. When studying the cross-reactions of hapten analogs, some haptens bind all antibodies, but with reduced K_A. Other hapten analogs reach a plateau of binding, since they fit some antibody-combining sites quite well but not others (see discussion of cross-reactivity in Chapter 12). Antibodies raised in different animals may show different cross-reactivities with related haptens. Even within a single animal, antibody affinity and specificity are known to increase over time following immunization under certain conditions (7). Thus any statements about the cross-reactivity of two haptens reflect both structural differences between the haptens which affect antigen–antibody fit and the diversity of antibody-binding sites present in a given antiserum.

Carbohydrate Antigens

The antigenic determinants of a number of biologically important substances consist of carbohydrates. These often occur as glycolipids or glycoproteins. Examples of the former include bacterial cell wall antigens and the major blood group antigens, whereas the latter group includes "minor" blood group antigens such as Rh. In addition, a number of spontaneously arising myeloma proteins have been found to show carbohydrate specificity, possibly reflecting the fact that carbohydrates are common environmental antigens. In the days prior to hybridoma technology, these carbohydrate specific myeloma proteins provided an important model for studying the reaction of antigen with a monoclonal antibody.

Empirically, the predominant antigenic determinants of polysaccharides often consist of short oligosaccharides (one to five sugars long) at the nonreducing end of the polymer chain (8). This situation is analogous to a hapten consisting of several sugar residues linked to a large nonantigenic polysaccharide backbone. The remainder of the polysaccharide is important for immunogenicity, just as the carrier molecule was important for haptens. In addition, branch points in the polysaccharide structure allow for multiple antigenic determinants to be attached to the same macromolecule. This is important for immunoprecipitation by lattice formation, as discussed in Chapter 12. Several examples illustrating structural studies of oligosaccharide antigens are given later.

The technique used most widely to analyze the antigenic determinants of polysaccharides is called hapten inhibition (8). In this method, the precipitation reaction between antigen and antibody is inhibited by adding short oligosaccharides. These oligosaccharides are large enough to bind with the same affinity and specificity as the polysaccharide, but because they are monomeric, no precipitate forms. As more inhibitor is added, fewer antibody-combining sites remain available for precipitation. Using antiserum specific for a single antigenic deter-

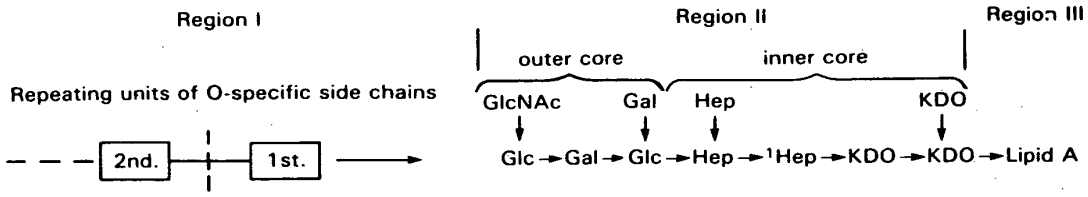
minant, it is often possible to block precipitation completely with a short oligosaccharide corresponding to the nonreducing end of the polysaccharide chain. Besides showing the "immunodominance" of the nonreducing end of the chain, this result also shows that the structure of the antigenic determinant of polysaccharides depends on the sequence of carbohydrates and their linkage, rather than their conformation. For inhibition by hapten to be complete, the antigen–antibody system studied must be made specific for a single antigenic determinant. For optimal sensitivity, the equivalence point of antigen and antibody should be used.

We illustrate the types of carbohydrate antigens encountered by examining three classic examples in more detail: the salmonella O antigens, the blood group antigens, and dextrans that bind to myeloma proteins.

Immunochemistry of Salmonella O Antigens

The antigenic diversity among numerous salmonella species resides in the structural differences of the lipopolysaccharide (LPS) component of the outer membrane (9). These molecules are the main target for anti-salmonella antibodies. The polysaccharide moiety contains the antigenic determinant, whereas the lipid moiety is responsible for endotoxin effects. The chemical structure of LPS can be divided into three regions (Fig. 1). Region I contains the antigenic O specific polysaccharide, usually made up of repeated oligosaccharide units, which vary widely among different strains. Region II contains an oligosaccharide "common core" shared among many different strains. Failure to synthesize region II oligosaccharide or to couple completed region I

A. Core Oligosaccharide Structure (Region II)



B. Oligosaccharide Antigens (Region I)

Group	Strain	Antigenic Determinants	Structure of Repeating Unit
A	*S.paratyphi* A	1,2,12	Glc, Par; 1,4 1,6 α; 1,3 α; Gal 1,2 α → Man 1,4 α → Rha 1,3 α; OAc
B	*S. typhimurium*	4,5,12	Glc 2-OAc-Abe; 1,4 α; 1,3 α; Gal 1,2 α → Man 1,4 α → Rha 1,3
D	*S. typhi*	9,12	(2-OAc-)Glc, Tyv; 1,4; 1,3 α; Gal 1,2 α → Man 1,4 α → Rha 1,3 α

FIG. 1. Structure of *Salmonella* lipopolysaccharide. Region I contains the unique O-antigen determinants, which consist of repeating units of oligosaccharides. These are attached to lipid moiety through the core polysaccharide. Three examples of oligosaccharide units are shown (9). (Part **A** adapted from ref. 8; part **B** based on ref. 9.)

indicate that there is variation in the size of different antigenic determinants of polysaccharides.

Genetic studies of the effect of phage lysogeny on salmonella polysaccharide antigens (10) have shown that sequential chemical modifications at the nonreducing end of the polysaccharide can cause stepwise changes in serotype (Table 4). Thus phage E15 converts *Salmonella anatum* O antigen from determinants 3,10 to 3,15, whereas subsequent lysogeny with phage E34 converts the O antigen to determinant 34. Hapten inhibition studies show completely different patterns for all three O antigens. The first lysogeny removes the terminal acetylation of galactose and changes the disaccharide bond from $\alpha(1 \rightarrow 6)$ to $\beta(1 \rightarrow 6)$. This removes determinant 10, while adding determinant 15. The subsequent lysogeny uses β-D-Gal as an acceptor for an additional D-Glu. This removes O antigen determinant 15 and adds determinant 34. In each case, removing an acetyl group and changing the first disaccharide linkage, or adding an additional sugar, changes the O antigen determinant, because the terminal sugar is immunodominant. The biochemical alteration induced by the phage is presumably due to the production of a glycosyltransferase adding the new sugar. This enzyme may be encoded by a phage gene or by a derepressed endogenous bacterial gene.

Blood Group Antigens

The major blood group antigens A and B were originally detected by the ability of serum from individuals lacking either determinant to agglutinate red blood cells bearing them (for reviews, see refs. 8 and 11–13). In addition, group O individuals have an H antigenic determinant that is not detectable on A or B type red blood cells, and individuals in all three groups may have additional determinants such as the Lewis (Le) antigens. Although the ABH and Le antigenic determinants are found on a carbohydrate moiety, the carbohydrate may occur in a variety of biochemical forms. On cell surfaces, they are either glycolipids that are synthesized within the cell (AB and H antigens) or glycoproteins taken up from serum (Lewis antigens). In mucinous secretions, such as saliva, they occur as glycoproteins. Milk, ovarian cyst fluid, and gastric mucosa are a source of soluble oligosaccharides containing blood group reactivity. In addition, these antigens occur frequently in other species, including about half of the bacteria in the normal flora of the gut (11). This widespread occurrence may account for the ubiquitous anti-AB reactivity of human sera, even in people never previously exposed to human blood group substances through transfusion or pregnancy.

The immunochemistry of these antigens was simplified greatly by the fact that antigenic oligosaccharides could be used for hapten inhibition studies. Group A oligosaccharides, for example, would inhibit the agglutination of group A red blood cells by anti-A antibodies. They could also inhibit the immunoprecipitation of group A-bearing glycoproteins by anti-A antibodies. Since the oligosaccharides are monomeric, their reaction with antibody does not form a precipitate but does block an antibody-combining site.

The inhibitory oligosaccharides from cyst fluid were purified and found to contain D-galactose, L-fucose, *N*-acetyl galactosamine, and *N*-acetylglucosamine. The most inhibitory oligosaccharides for each antigen are indicated in Fig. 2. As can be seen in the figure the ABH and Le antigens all share a common oligosaccharide core sequence, and the antigens appear to differ from each other by the sequential addition of individual sugars at the end or at branch points. Besides hapten inhibition, other biochemical data support this relationship among

TABLE 4. *Stepwise changes in serotype of S. anatum due to phage; lysogeny represents successive alterations at the nonreducing end*

Hapten concentration causing 50% inhibition (mM)	Antigen–antibody system tested		
	10.anti-10	15.anti-15	34.anti-34
AcO-Gal Man L-Rham	0.013	N.D.[a]	N.D.[a]
Gal-Man L-Rham	>1.6	0.005	0.16
Gal-Man	—[b]	0.027	⩾0.2
Man L-Rham	—[b]	0.083	⩾0.2
Glu-Gal-Man	—[b]	0.027	0.05
Glu-Gal	—[b]	1.0	0.10
Deduced structure			
Antigen 10	AcO-Galα(1 → 6)Manα(1 → 4) L-Rham		
Phage E_{15} ↓			
Antigen 15	Galβ(1 → 6)Manα(1 → 4) L-Rham		
Phage E_{34} ↓			
Antigen 34	α-D-Gluα(1 → 4)Galβ(1 → 6) Man		

From ref. 10, with permission.
[a] N.D., not done.
[b] No inhibition at any concentration tested.

TABLE 2. *Salmonella O antigen serotyping*

Salmonella strain	Sero group	O antigenic determinants
S. paratyphi A	A	1, 2, 12
S. paratyphi B	B	1, 4, 5, 12
S. typhi	D	9, 12

Antiserum	Absorbed	Tested on	Single determinant measured
Anti-*S. typhi*	—	*S. paratyphi B*	12
Anti-*S. typhi*	*S. paratyphi A*	*S. typhi*	9

From ref. 8, with permission.

polysaccharide to the growing region II core results in R (rough) mutants, which have "rough" colony morphology and lack the O antigen. Region III is the lipid part, called lipid A, which is shared among all salmonellae and serves to anchor LPS on the outer membrane. Early immunologic attempts to classify the O antigens of different salmonellae revealed a large number of cross-reactions between different strains. These were detected by preparing antiserum to one strain of salmonella and using it to agglutinate bacteria of a second strain. Each cross-reacting determinant was assigned a number, and each strain was characterized by a series of O antigen determinants (in aggregate, the "serotype" of the strain) based on its pattern of cross-reactivity. Each strain was classified within a group, based on sharing a strong O determinant. For example, group A strains share determinant 2, whereas group B strains share determinant 4 (Table 2). However, within a group, each strain possesses additional O determinants, which serve to differentiate it from other members of that group. Thus determinant 2 coexists with determinants 1 and 12 on *Salmonella paratyphi A*. This problem of cross-reactivity based on sharing of a subset of antigenic determinants is commonly encountered in complex antigen–antibody systems. The problem may be simplified by making antibodies monospecific for individual antigenic determinants. To do this, antibodies are absorbed to remove irrelevant specificities, or cross-reactive strains are chosen that share only a single determinant with the immunizing strain. The reaction of each determinant with its specific antibody can be thought of as an antigen–antibody system. Thus for the strains shown in Table 2, antiserum to *Salmonella typhi* (containing anti-9 and anti-12 antibodies) may be absorbed with *S. paratyphi A* to remove anti-12, leaving a reagent specific for antigen 9 (Table 2). Alternatively, the unabsorbed antiserum may be used to study the system antigen 12–anti-12 by allowing it to agglutinate *S. paratyphi B*, which shares only antigen 12 with the immunogen. Because the other determinants on *S. paratyphi B* were absent from the immunizing strain, the antiserum contains no antibodies to them.

Once the antigen–antibody reaction is made specific for a single determinant, a variety of oligosaccharides can be added to test for hapten inhibition. Since the O antigens contain repeating oligosaccharide units, it is often possible to obtain model oligosaccharides by mild chemical or enzymatic degradation of the LPS polysaccharide itself. Once the most inhibitory oligosaccharide is found, its chemical structure is determined. Alternatively, a variety of synthetic mono-, di-, tri-, and oligosaccharides are tested for hapten inhibition of precipitation. For example, as shown in Table 3, antigen 1–anti-1 antibody precipitation is inhibited by methyl-α-D-glucoside. Therefore various disaccharides incorporating this structure were tested, of which α-D-Glu(1 → 6)D-Gal was the most inhibitory. Then various trisaccharides incorporating this sequence were tested. The results indicate the sequence and size of the determinant recognized by anti-1 antibodies to be a disaccharide with the above structure. The test sequences can be guessed by analyzing the oligosaccharide breakdown products of the lipopolysaccharide, which include tetramers of D-Glu-D-Gal-D-Man-L-Rham. The results in Table 3 also suggest that the difference between determinants 1 and 19 is the length of oligosaccharide recognized by antibodies specific for each determinant. This hypothesis is supported by the observation that determinant 1 is found in some strains with, and in other strains without, determinant 19; whereas determinant 19 is always found with determinant 1. As shown in the table, determinant 19 requires the full tetrasaccharide for maximal hapten inhibition, including the sequence coding for determinant 1. Besides identifying the antigenic structures, these results

TABLE 3. *Analysis of salmonella O-antigen structure by hapten inhibition*

Maximum inhibition by hapten (%)	Antigen system	
	1.anti-1	19.anti-19
D-Glu	—	0
Me-α-D-Glu	35	10
α-D-Glu(1 → 6)-D-Gal	80	25
Glu.Gal.Man	80	70
Glu.Gal.Man.L-Rham	>70	>70
Deduced structure	α-D-Glu(1 → 6)-D-Gal	D-Glu-D-Gal-D-Man-L-Rham

From ref. 8, with permission.

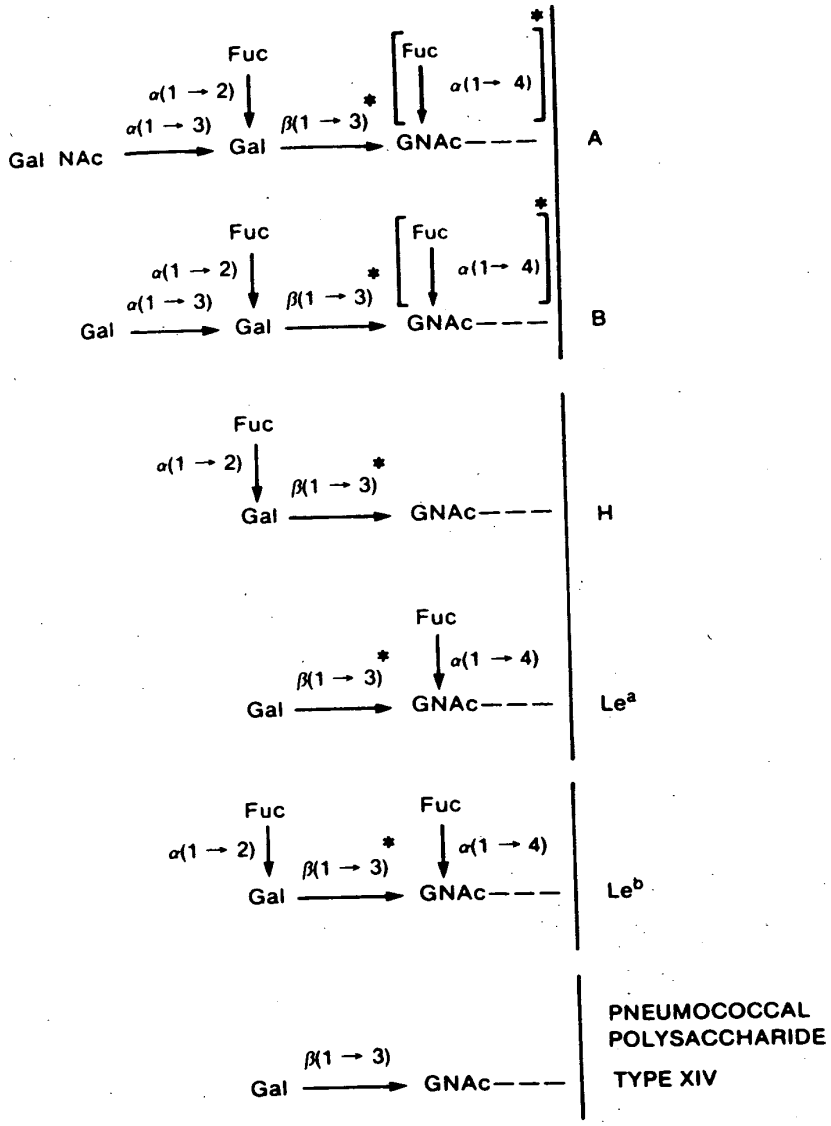


FIG. 2. Oligosaccharide chain specificity. Structure of the ABH and Le blood group antigens, as determined by hapten inhibition studies (8,12). There are two variants of each of these determinants. In type 1, the Gal-GNAc linkage is $\beta(1 \rightarrow 3)$, whereas in type 2, the Gal–GNAc linkage is $\beta(1 \rightarrow 4)$. In addition, there is heterogeneity in the A and B antigens with respect to the presence of the Le fucose attached to the GNAc. In the molecules that contain the extra fucose, when the Gal-GNAc linkage is $\beta(1 \rightarrow 3)$ (type 1), the fucose must be linked $\alpha(1 \rightarrow 4)$, whereas the type 2 molecules, with the $\beta(1 \rightarrow 4)$ Gal-GNAc linkage, contain $\alpha(1 \rightarrow 3)$-linked fucose. The *asterisks* indicate the sites of this variability in linkage.

the different determinants. Enzymatic digestion of A, B, or H antigens yields a common core oligosaccharide from each. This product cross-reacts with antiserum specific for pneumococcal polysaccharide type XIV, which contains structural elements shared with blood group determinants, as shown at the bottom of Fig. 2. In addition, this structure, known as precursor substance, has been isolated from ovarian cyst fluid.

Starting from precursor substance, the H determinant results from the addition of L-fucose to galactose, whereas Le^a determinant results from the addition of L-fucose to *N*-acetylglucosamine, and Le^b from the addition of L-fucose to both sugars. Addition of *N*-acetylgalactosamine to H substance produces the A determinant, whereas addition of galactose produces the B determinant, in each case blocking reactivity of the H determinant.

The genetics of ABH and Le antigens is explained by this sequential addition of sugars via glycosyltransferases. The allelic nature of the AB antigens is explained by the addition of *N*-acetylgalactosamine, galactose, or nothing to the H antigen. The rare inherited trait of inability to synthesize the H determinants from precursor substance (Bombay phenotype) also blocks the expression of A and B antigens, since the A and B transferases lack an acceptor substrate. However, the appearance of the Lewis[a] antigen (Le^a) on red cells is independent of H antigen synthesis. Its structure, shown in Fig. 2, can be derived directly from precursor substance without going through an H antigen intermediate. Comparing different

individuals, the appearance of Le^a antigen on red blood cells correlates with its presence in saliva, since the Le^a antigen is not an intrinsic membrane component but must be absorbed from serum glycoproteins, which, in turn, depend on secretion. In addition to the independent synthetic pathway, the secretion of Le^a antigen is also independent of the secretory process for ABH antigens. Therefore salivary nonsecretors of ABH antigens (which occur in 20% of individuals) may still secrete Le^a antigen if they have the fucosyl transferase encoded by the *Le* gene. In contrast, salivary secretors of ABH may produce Le^b antigen through the action of the H antigen specific fucosyl transferase on the Le^a antigen followed by secretion via the secretory system for ABH antigens. Because it becomes the substrate for additional biochemical steps, Le^a antigen secretion is reduced, and it is usually undetectable on red blood cells of ABH (and Le^b) secretors. Conversely, Le^b is present only in ABH secretors.

Dextran-Binding Myeloma Proteins

Since polysaccharides are common environmental antigens, it is not surprising that randomly induced myeloma proteins were frequently found to have carbohydrate specificities. Careful studies of these monoclonal antibodies support the clonal expansion model of antibody diversity: heterogeneous antisera behave as the sum of many individual clones of antibody with respect to affinity and specificity. In the case of the IgAκ myeloma proteins W3129 and W3434, both antibodies were found to be specific for dextrans containing α-glu $(1 \rightarrow 6)$glu bonds (13). Hapten inhibition with a series of mono- or oligosaccharides of increasing chain length indicated that the percentage of binding energy derived from the reaction with one glucose was 75%, two glucoses 95%, three 95% to 98%, and four 100%. This suggests that most binding energy between antidextran antibodies and dextran derives from the terminal monosaccharide, and that oligosaccharides of chain length 4 to 6 commonly fill the antibody-combining site. Human antidextran antisera behaved similarly, with tetrasaccharides contributing 95% of the binding energy. These experiments provided the first measure of the size of an antigenic determinant, 4 to 6 residues (14,15). In addition, as was observed for antisera, binding affinity of myeloma proteins was highly sensitive to modifications of the terminal sugar and highly specific for $\alpha(1 \rightarrow 6)$ versus $\alpha(1 \rightarrow 3)$ glycosidic bonds. However, modification of the third or fourth sugar of an oligosaccharide had relatively less effect on hapten inhibition of either myeloma protein or of antisera reacting with dextran.

Studies with additional dextran-binding myeloma proteins (16) revealed that not all antipolysaccharide monoclonal antibodies are specific for the nonreducing end, as exemplified by QUPC 52. Competitive inhibition with mono- and oligosaccharides revealed that less than 5% of binding energy derived from mono- or disaccharides, 72% from trisaccharides, 88% from tetrasaccharides, and 100% from hexasaccharides, in marked contrast to other myeloma proteins. A second distinctive property of myeloma protein QUPC 52 was its ability to precipitate unbranched dextran of chain length 200. Since the unbranched dextran has only one nonreducing end, and since the myeloma protein has only one specificity, lattice formation by binding to the nonreducing ends is impossible and precipitation must be explained by binding some other determinant. Therefore QUPC 52 appears to be specific for internal oligosaccharide units of 3 to 7 chain length. W3129 is specific for end determinants and will not precipitate unbranched dextran chains. Antibodies precipitating linear dextran were also detected in six antidextran human sera, comprising 48% to 90% of the total antibodies to branched chain dextran. Thus antidextrans can be divided into those specific for terminal oligosaccharides and those specific for internal oligosaccharides; monoclonal examples of both types are available, and both types are present in human immune serum. Cisar et al. (16) speculated as to the different topology of the binding sites of W3129 or QUPC 52 necessary for terminal or internal oligosaccharide specificity. Both terminal and internal oligosaccharides have nearly identical chemical structures, differing at a single C—OH or glycoside bond. Perhaps the terminal oligosaccharide specificity of W3129 is due to the shape of the antibody-combining site—a cavity into which only the end can fit; whereas the internal oligosaccharide-binding site of QUPC 52 could be a surface groove in the antibody, which would allow the rest of the polymer to protrude out at both ends. A more definitive answer depends on x-ray crystallographic studies of the combining sites of monoclonal antibodies with precisely defined specificity, performed with antigen occupying the binding site.

With the advent of hybridoma technology, it became possible to produce monoclonal antibodies of any desired specificity. Immunizing mice with nearly linear dextran (the preferred antigen of QUPC 52), followed by fusion and screening (with linear dextran) for dextran-binding antibodies, yielded 12 hybridomas (17), all with specificity similar to QUPC 52. Oligosaccharide inhibition of all 12 monoclonals showed considerable increments in affinity up to hexasaccharides, with little affinity for disaccharides and only 49% to 77% of binding energy derived from trisaccharides (59). Second, all 12 monoclonals had internal $\alpha(1 \rightarrow 6)$ dextran specificity, since they could all precipitate linear dextran. Third, 9 out of 11 BALB/c monoclonals shared cross-reactive idiotype with QUPC 52, whereas none shared idiotype with W3129 (18). These data support the hypothesis that different antibodies with similar specificity and similar

groove-type sites may be derived from the same family of germline V_H genes bearing the QUPC 52 idiotype (18).

The large number of environmental carbohydrate antigens and the high degree of specificity of antibodies elicited in response to each carbohydrate antigen suggest that a tremendous diversity of antibody molecules must be available, from which some antibodies can be selected for every possible antigenic structure. In order to regulate such a diverse system, a network theory has been proposed, in which antibodies are themselves recognized as antigenic (see Chapters 12 and 24, and ref. 19), and the response to streptococcal polysaccharide is a leading example in which anti-idiotypic antibodies can be shown to regulate the response to antigen (20).

Recent studies of a series of 17 monoclonal anti-α (1 → 6) dextran hybridomas (21,22) have investigated whether the binding sites of closely related antibodies would be derived from a small number of variable region genes, for both heavy and light chains, or whether antibodies of the same specificity could derive from variable region genes with highly divergent sequences. Each monoclonal had a groove-type site that could hold six or seven sugar residues (with one exception), based on inhibition of immunoprecipitation by different length oligosaccharides. Thus, unlike monoclonals to haptenated proteins, the precise epitope could be well characterized and was generally quite similar among the entire series.

Studies of the V_κ sequences revealed that only three V_κ groups were used in these hybridomas. Use of each V_κ group correlated with the particular antigen used to immunize the animals, whether linear dextran or short oligosaccharides, so that 10 of the monoclonals from mice immunized the same way all used the same V_κ.

In contrast, the 17 V_H chains were derived from at least five different germline genes from three different V_H gene families (23). The two most frequently used germline V_H genes were found in seven and five monoclonals, respectively, with minor variations explainable by somatic mutations. Once again, V_H gene usage correlated with size of the antigen used to immunize, although the length of each CDR did not correlate with the size of the groove-type binding site. The remarkable finding is that very different V_H chains (about 50% homologous) can combine with the same V_K to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. A similar phenomenon can also occur when different V_H sequences combine with different V_K sequences to produce antibodies with very similar properties. This is a result of the fact that dextran binding depends on the antigen fitting into the groove and interacting favorably with the residues forming the sides and bottom of the groove. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. Similar results have been reported in other antigen–antibody systems, such as phenyloxazolone (24).

More recently, these studies were expanded to include 34 groove-type monoclonal anti-α(1 → 6) dextran-binding hybridomas (25), of which 10 used heavy chain V_H19.1.2 and eleven used V_H9.14.7. Starting with different V_H genes, these two families of monoclonals provide an experiment of nature concerning the ability of each V_H gene to combine with different light chain V_K and J_K genes, as well as heavy chain D and J_H genes to produce a groove-shaped binding site of a given specificity. In every one of these 21 monoclonals, the same light chain V_K-Ox1 gene was used, but the V_H19 family used a single J_K sequence exclusively (J_K2), while the V_H9 family included all four of the active J_K segments (J_K1, 2, 4, and 5). Similarly, the heavy chain J_H sequences of the V_H19 family were all of a single type (J_H3), while those of the V_H9 family included three types (J_H1, 2, and 3). A single D region was used by both families (DFL16.1), but the junctional sequences between V_H–D and D–J_H were different, with the V_H19 using minimal substitutions, and the V_H9 allowing more variability in junctional sequences, depending on the size of the J_H with which it was joining. Although the amino acid sequences of these two V_H genes are 73% identical, they use markedly different strategies to arrive at the same groove-type binding site with nearly identical size and specificity. The results suggest that the two heavy chain variable regions, perhaps due to their conformation, may place different structural constraints on which minigene components can successfully contribute to forming a particular site. Two different strategies for generating antibody specificity are apparent, even though the same V_K and D minigenes were used by both families. For the V_H19 family, point mutations in the CDR2 generated the α(1 → 6) dextran specificity, while the rest of the structure was held constant. For the V_H9 family, a wide variety of J_H, J_K, and V_H–D and D–J_H sequences were used to generate the groove-type site. These two blueprints for constructing a binding site may also reflect distinct cellular pathways for generating antibody diversity.

Protein and Polypeptide Antigenic Determinants

Like the proteins themselves, the antigen determinants of proteins consist of amino acid residues in a particular three-dimensional array. The residues that make contact with complementary residues in the antibody-combining site are called contact residues. To make contact, of course, these residues must be exposed on the surface of the protein, not buried in the hydrophobic core. Since the complementarity-determining residues in the hypervariable regions of antibodies have been found to span as much as 30 to 40 Å × 15 to 20 Å × 10 Å (D. R. Davies, *personal communication*), these contact

residues comprising the antigenic determinant may cover a significant area of protein surface, as now measured in a few cases by x-ray crystallography of antibody–protein antigen complexes (26–28). Looked at from another point of view, the size of the combining sites has been estimated using simple synthetic oligopeptides of increasing length, such as oligolysine. In this case, a series of elegant studies (29–31) suggested that the maximum length of chain a combining site could accommodate was six to eight residues, corresponding closely to that found earlier for oligosaccharides (14,15), discussed previously.

Several types of interactions contribute to the binding energy. Many of the amino acid residues exposed to solvent on the surface of a protein antigen will be hydrophilic. These are likely to interact with antibody contact residues via polar interactions. For instance, an anionic glutamic acid carboxyl group may bind to a complementary cationic lysine amino group on the antibody, or vice versa; or a glutamine amide side chain may form a hydrogen bond with the antibody. However, hydrophobic interactions can also play a major role. Proteins cannot exist in aqueous solution as stable monomers with too many hydrophobic residues on their surface. Those hydrophobic residues that are on the surface can contribute to binding to antibody for exactly the same reason. When a hydrophobic residue in a protein antigenic determinant or, similarly, in a carbohydrate determinant (8) interacts with a corresponding hydrophobic residue in the antibody-combining site, the water molecules previously in contact with each of them are excluded. The result is a significant stabilization of the interaction. A thorough review of these aspects of the chemistry of antigen–antibody binding has recently been published (32).

Mapping Epitopes: Conformation Versus Sequence

The other component that defines a protein antigenic determinant, besides the amino acid residues involved, is the way these residues are arrayed in three dimensions. Since the residues are on the surface of a protein, we can also think of this component as the topography of the antigenic determinant. Sela (33) divided protein antigenic determinants into two categories, sequential and conformational, depending on whether the primary sequence or the three-dimensional conformation appeared to contribute the most to binding. On the other hand, since the antibody-combining site has a preferred topography in the native antibody, it would seem *a priori* that some conformations of a particular polypeptide sequence would produce a better fit than others and therefore would be energetically favored in binding. Thus conformation or topography must always play some role in the structure of an antigenic determinant.

Moreover, when one looks at the surface of a protein in a space-filling model, one cannot ascertain the direction of the backbone or the positions of the helices (contrast Figs. 3 and 4). It is hard to recognize whether two residues that are side by side on the surface are adjacent on the polypeptide backbone or whether they come from different parts of the sequence and are brought together by the folding of the molecule. If a protein maintains its native conformation when an antibody binds, then it must similarly be hard for the antibody to discriminate between residues that are covalently connected directly and those connected only through a great deal of intervening polypeptide. Thus the probability that an antigenic determinant on a native globular protein consists of only a consecutive sequence of amino acids in the primary structure is likely to be rather small. Even if most of the determinant were a continuous sequence, other nearby residues would probably play a role as well. Only if the protein were cleaved into fragments before the antibodies were made would there be any reason to favor connected sequences.

This concept was analyzed and confirmed quantitatively by Barlow et al. (39), who examined the atoms lying within spheres of different radii from a given surface atom on a protein. As the radius increases, the probability that all the atoms within the sphere will be from the same continuous segment of protein sequence decreases rapidly. Correspondingly, the fraction of surface atoms that would be located at the center of a sphere containing only residues from the same continuous segment falls dramatically as the radius of the sphere increases. For instance, for lysozyme, with a radius of 8 Å, fewer than 10% of the surface residues would lie in such a "continuous patch" of surface. These are primarily in regions that protrude from the surface. With a radius of 10 Å, almost none of the surface residues fall in the center of a continuous patch. Thus for a contact area of about 20 Å × 25 Å, as found for a lysozyme–antibody complex studied by x-ray crystallography, none of the antigenic sites could be completely continuous segmental sites.

Antigenic sites consisting of amino acid residues that are widely separated in the primary protein sequence but brought together on the surface of the protein by the way it folds in its native conformation have been called "assembled topographic" sites (40,41) because they are assembled from different parts of the sequence and exist only in the surface topography of the native molecule. By contrast, the sites that consist of only a single continuous segment of protein sequence have been called "segmental" antigenic sites (40,41).

In contrast to T cell recognition of "processed" fragments retaining only primary and secondary structures, the evidence is overwhelming that most antibodies are made against the native conformation when the native protein is used as immunogen. For instance, antibodies to native staphylococcal nuclease were found to have about a 5000-fold higher affinity for the native protein

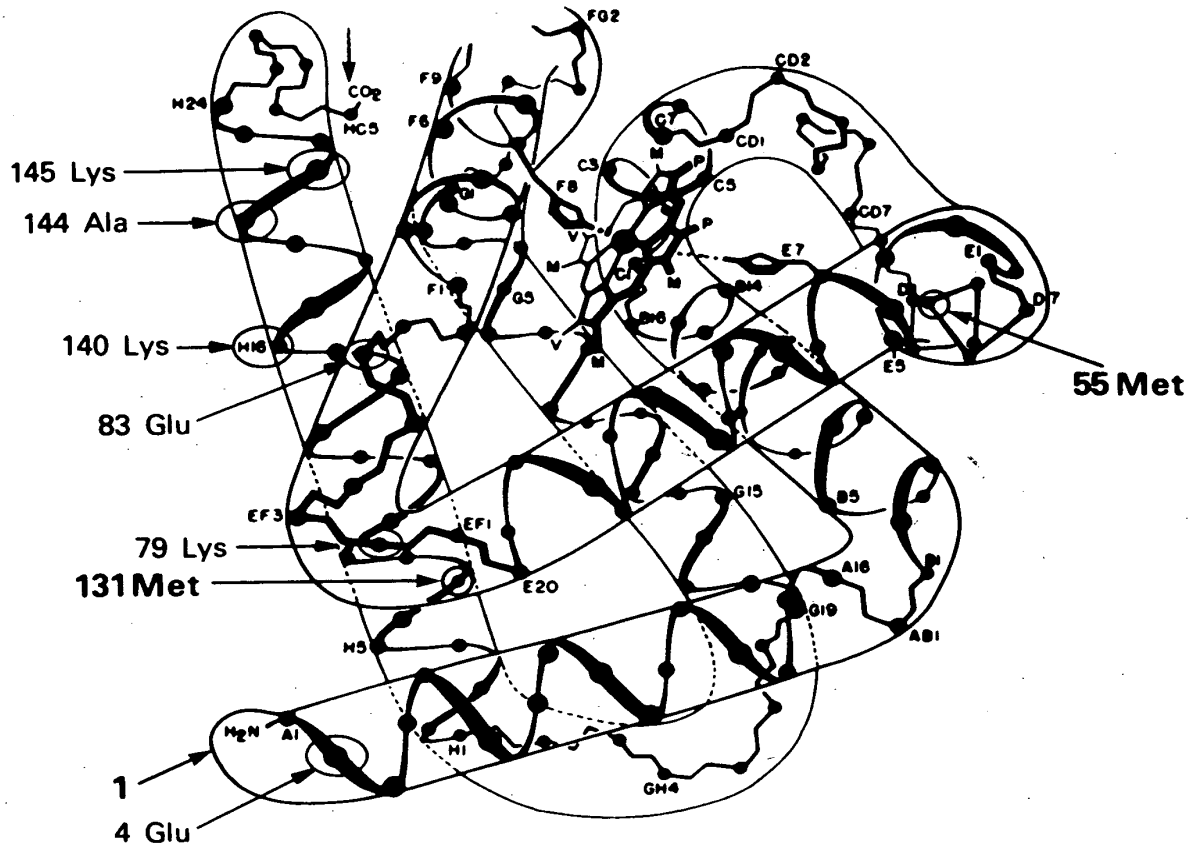


FIG. 3. Artist's representation of the polypeptide backbone of sperm whale myoglobin in its native three-dimensional conformation. The α helices are labeled A through H from the amino terminal to the carboxyl terminal. Side chains are omitted, except for the two histidine rings (F8 and E7) involved with the heme iron. Methionines at positions 55 and 131 are the sites of cleavage by cyanogen bromide (CNBr), allowing myoglobin to be cleaved into three fragments. Most of the helicity and other features of the native conformation are lost when the molecule is cleaved. A less drastic change in conformation is produced by removal of the heme to form apomyoglobin, since the heme interacts with several helices and stabilizes their positions relative to one another. The other labeled residues (4 Glu, 79 Lys, 83 Glu, 140 Lys, 144 Ala, and 145 Lys) are residues that have been found to be involved in antigenic determinants recognized by monoclonal antibodies (34). Note that cleavage by CNBr separates Lys 79 from Gly^{34} 4 and separates Glu 83 from Ala 144 and Lys 145. The "sequential" determinant of Koketsu and Atassi (35) (residues 15 to 22) is located at the elbow, **lower right,** from the end of the A helix to the beginning of the B helix. (Adapted from ref. 36.)

than for the corresponding polypeptide on which they were isolated (by binding to the peptide attached to Sepharose) (42). An even more dramatic example is that demonstrated by Crumpton (43) for antibodies to native myoglobin or to apomyoglobin. Antibodies to native ferric myoglobin produced a brown precipitate with myoglobin, an indication that the heme was still in the protein in what was, at least approximately, its native environment. Such antibodies did not bind well to the apomyoglobin, which, without the heme, has a slightly altered conformation. On the other hand, antibodies to the apomyoglobin, when mixed with native (brown) myoglobin, produced a white precipitate. These antibodies so strongly favored the conformation of apomyoglobin, from which the heme was excluded, that they trapped those molecules that vibrated toward that conformation and pulled the equilibrium state over to the apo form. One could almost say, figuratively, that the antibodies squeezed the heme out of the myoglobin. Looked at thermodynamically, it is clear that the conformational preference of the antibody for the apo versus native forms, in terms of free energy, had to be greater than the free energy of binding of the heme to myoglobin. Thus, in general, antibodies are made that are very specific for the conformation of the protein used as immunogen.

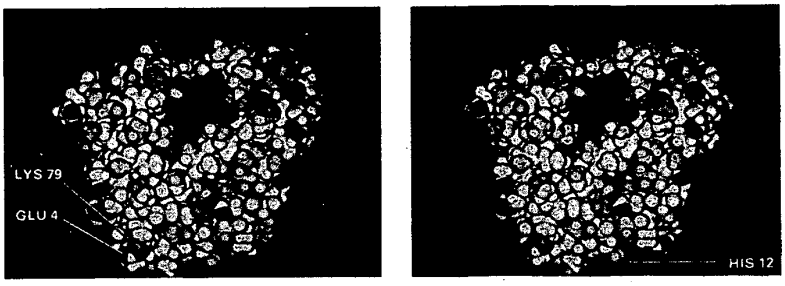


FIG. 4. Stereoscopic views of a computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano (37) x-ray diffraction coordinates. This orientation, which corresponds to that in Fig. 3, is arbitrarily designated the "front view." The computer method was described by Feldmann et al. (38). The heme and aromatic carbons are *shaded darkest,* followed by carboxyl oxygens, then other oxygens, then primary amino groups, then other nitrogens, and finally side chains of aliphatic residues. The backbone and the side chains of nonaliphatic residues, except for the functional groups, are shown in *white.* Note that the direction of the helices is not apparent on the surface, in contrast to the backbone drawing in Fig. 3. The residues Glu 4, Lys 79, and His 12 are believed to be part of a topographic antigenic determinant recognized by a monoclonal antibody to myoglobin (34). This stereo pair can be viewed in three dimensions using an inexpensive stereoviewer such as the "stereoscopes" sold by Abrams Instrument Corp., Lansing, MI, or Hubbard Scientific Co., Northbrook, IL. (Adapted from ref. 34.)

A number of methods have been used to identify the antigenic determinants bound by particular antibodies made against a protein. Binding to cleavage fragments and short synthetic peptides from the protein sequence has been the most widely used approach. The synthetic peptides may be made by conventional solid-phase peptide synthesis (44) or by methods designed to make large numbers of peptides for screening. In one such method, multiple peptides are made simultaneously in separate polypropylene mesh "tea-bags" that can be put through the common steps in the sequence together and separated only for the different amino acid coupling steps (45). In another method, the peptides are synthesized on the tips of plastic pins inserted in the wells of 96-well microtiter plates in such a way that these can then be used for solid-phase binding assays of antibodies without ever cleaving the peptide off the plastic support (46). These two methods especially lend themselves to studying multiple variants of the natural sequence to identify the residues critical for antibody binding. Usually, the longer the peptides, the more that specificity can be confidently determined, as short peptides of only six to eight amino acid residues often manifest nonspecific binding (47). If the synthetic peptides correspond to segments of the protein antigen sequence, as is most common, then the use of peptides is limited to identifying the structures bound by antibodies specific for segmental antigenic sites.

To identify assembled topographic sites, more complex approaches have been necessary. The earliest was the use of natural variants of the protein antigen with known amino acid substitutions, where such evolutionary variants exist (40). Thus substitution of different amino acids in proteins in the native conformation can be examined. The use of this method, which is illustrated later, is limited to studying the function of amino acids that vary among homologous proteins, that is, those that are polymorphic. It may now be extended to other residues by use of site-directed mutagenesis. A second method is to use the antibody that binds to the native protein to protect the antigenic site from modification (48) or proteolytic degradation (49). A related but less sensitive approach makes use of competition with other antibodies (50–52). A third approach, taking advantage of the capability of producing thousands of peptides on a solid-phase surface for direct binding assays (46), is to study binding of a monoclonal antibody to every possible combination of six amino acids (46). If the assembled topographic site can be mimicked by a combination of six amino acids not corresponding to any continuous segment of the protein sequence but structurally resembling a part of the surface, then one can produce a "mimotope" defining the specificity of that antibody (46).

Myoglobin also serves as a good model protein antigen for studying the range of variation of antigenic determinants from those that are more sequential in nature to those that do not even exist without the native conformation of the protein (Fig. 3). A good example of the first, more segmental type of determinant is that consisting of residues 15 to 22 in the amino terminal portion of the molecule. Crumpton and Wilkinson (53) first discovered that the chymotryptic cleavage fragment consisting of residues 15 to 29 had antigenic activity for antibodies raised to either native or apomyoglobin. Synthetic peptides corresponding to the shorter sequence 15 to 22 were then found by two groups (35,54) to bind antibod-

ies made to native sperm whale myoglobin, even though the synthetic peptides were only 7 to 8 residues long. Peptides of this length do not spend much time (in solution) in a conformation corresponding to that of the native protein. On the other hand, these synthetic peptides had a several hundred-fold lower affinity for the antibodies than did the native protein. Thus, even if most of the determinant was included in the consecutive sequence 15 to 22, the antibodies were still much more specific for the native conformation of this sequence than for the random conformation peptide. Moreover, there was no evidence to exclude the participation of other residues, nearby on the surface of myoglobin but not in this sequence, in the antigenic determinant.[1]

A good example of the importance of secondary structure is the case of the loop peptide (residues 64 to 80) of hen egg-white lysozyme (59). This loop in the protein sequence is created by the disulfide linkage between cysteine residues 64 and 80 and has been shown to be a major antigenic determinant for antibodies to lysozyme (59). The isolated peptide 60 to 83, containing the loop, binds antibodies with high affinity, but opening of the loop by cleavage of the disulfide bond destroys most of the antigenic activity for antilysozyme antibodies (59).

At the other end of the range of conformational requirements are those determinants involving residues far apart in the primary sequences that are brought close together on the surface of the native molecule by its folding in three dimensions. Myoglobin also provides a good example of these determinants, which are called assembled topographic determinants (40,41). Of six monoclonal antibodies to sperm whale myoglobin studied by Berzofsky et al. (34,60), none bound to any of the three cyanogen bromide cleavage fragments of myoglobin that together span the whole sequence of the molecule. Therefore these monoclonal antibodies (all with affinities between 2×10^8 and 2×10^9 M^{-1}) were all highly specific for the native conformation. These were studied by comparing the relative affinities for a series of native myoglobins from different species with the known amino acid sequences of these myoglobins. With the myoglobins available, this approach allowed the definition of some of the residues involved in binding to three of these antibodies. The striking result was that two of these three monoclonal antibodies were found to recognize topographic determinants, as defined previously. One recognized a determinant including Glu 4 and Lys 79, which are on the A helix and E–F corner of the myoglobin molecule but come within about 2 Å of each other to form a salt bridge in the native molecule (Fig. 4). The other antibody recognized a determinant involving Glu 83 in the E–F corner, and Ala 144 and Lys 145 on the H helix of the myoglobin molecule (Fig. 5). Again, these are far apart in the primary sequence but are brought within 12 Å of each other by the folding of the molecule in its native conformation. Similar examples have recently been reported for monoclonal antibodies to human myoglobin (61) and to lysozyme (50). Other examples of such conformation-dependent antigenic determinants have been suggested using conventional antisera to such proteins as insulin (62), hemoglobin (63), tobacco mosaic virus (64), and cytochrome *c* (65). Moreover, the crystallographic structures of lysozyme–antibody (26,28) and neuraminidase–antibody (27) complexes show clearly that, in both cases, the epitope bound is an assembled topographic site.

How frequent are antibodies specific for topographic determinants compared to those that bind consecutive sequences when conventional antisera are examined? This question was studied by Lando et al. (66), who passed goat, sheep, and rabbit antisera to sperm whale myoglobin over columns of Sepharose-coupled cyanogen bromide cleavage fragments of myoglobin, together spanning the whole sequence. The antisera were passed sequentially, and repeatedly, over each of the three columns until no more antibodies could be removed. Nevertheless, 30% to 40% of the antibodies originally present in each serum remained after this treatment. These antibodies still bound to the native myoglobin molecule with high affinity but did not bind to any of the fragments in solution by radioimmunoassay. Thus, in four of four antimyoglobin sera tested, 60% to 70% of the antibodies could bind peptides and 30% to 40% could bind only native-conformation intact protein.

On the basis of studies such as these, it has been suggested that much of the surface of a protein molecule may be antigenic (40,67) but that the surface can be divided up into antigenic domains (34,57,58,61). Each of these domains consists of many overlapping determinants recognized by different antibodies.

An additional interesting point can be made from the above studies about the topography of protein antigenic determinants. If one examines the topographic determinant consisting of sperm whale myoglobin residues 83, 144, and 145, shown in stereo in Fig. 5, it is apparent that they are on both lips of a deep crevice or concavity in the protein surface. It is possible, although not yet demonstrated, that a complementary protuberance in the antibody-combining site actually inserts into this cavity. From the studies of myeloma proteins that bind small haptens or carbohydrates, we are accustomed to think of the antigen being engulfed by a cavity or crevice

[1] This is the only segmental antigenic determinant of myoglobin that has clearly been confirmed by more than one independent group of investigators. Crumpton and Wilkinson (53) did measure antigenic activity for a chymotryptic fragment 147 to 153 that overlaps one of the other reported sequential determinants (55). However, two of the other reported sequential determinants (55), corresponding to residues 56 to 62 and 94 to 100, have not been reproducible when tested with other antisera, even raised in the same species (56). For related studies, see refs. 57 and 58.

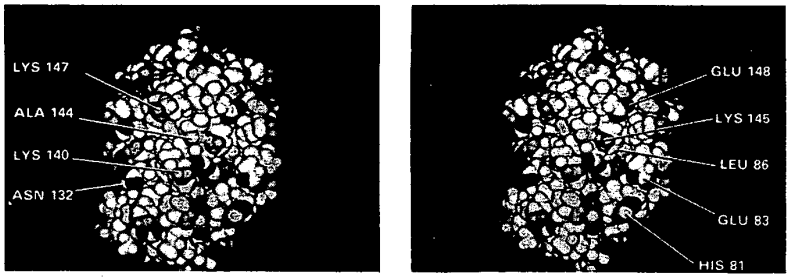


FIG. 5. Stereoscopic view of computer-generated space-filling model of the left view of sperm whale myoglobin, turned 90° relative to the view in Fig. 4. Methods and shading are as indicated in Fig. 4. The residues Glu 83, Ala 144, and Lys 145 are believed to be part of a topographic antigenic determinant recognized by a second monoclonal antibody to myoglobin (34). Note the concavity in the surface of the molecule in the middle of this determinant, between Glu 83 and Ala 144/Lys 145. (Adapted from ref. 34.)

on the antibody (68). However, for globular protein antigens binding to globular protein antibodies, the situation is more structurally symmetrical (and antigen–antibody binding is also thermodynamically symmetrical). Thus it is just as possible for a convexity on the antibody to insert into a concavity on the antigen as it is for the more conventional model to occur of a convexity on the antigen inserting into a concavity on the antibody. Now that monoclonal antibodies specific for protein antigens are available, we may encounter both types of cases. The determinant depicted in Fig. 5 might be such a case. In the three published crystal structures of protein antigen–antibody complexes, the contact surfaces were broad, with local complementary pairs of concave and convex regions in both directions (26–28). However, when we limit ourselves to antigenic sites defined with short peptides, which tend to identify sites that protrude from the surface of the antigen (39,69), we are likely to see a bias toward situations in which the antigen is convex and the antibody surface concave.

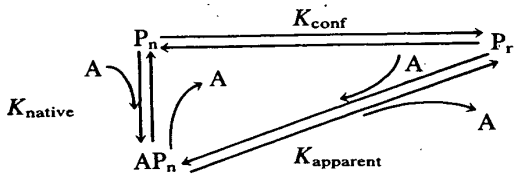
Further information on the subjects discussed in this section is available in the reviews by Sela (33), Crumpton (43), Reichlin (70), Kabat (68), Benjamin et al. (40), Berzofsky (41), and Getzoff et al. (32).

Conformational Equilibria of Protein and Peptide Antigenic Determinants

We have already referred to the fact that antibodies to a native protein have higher affinity for the native conformation than for other conformations of fragments or denatured molecules. Similarly, antibodies raised against fragments or denatured molecules generally have higher affinities for these forms than for the native conformation. In this section we discuss possible mechanisms for these affinity differences and explore how these can be used to advantage to study the conformational equilibria of proteins and peptides.

There are several possible mechanisms to explain why an antibody specific for a native protein will bind a peptide fragment in random conformation with lower affinity. Of course, the peptide may not contain all the contact residues of the antigenic determinant, so that the binding energy would be lower. However, for cases in which all the residues in the determinant are present in the peptide, several mechanisms still remain. First, the affinity may be lower because the topography of the residues in the peptide may not produce as complementary a fit in the antibody-combining site as the native conformation would. Second, it is possible that the apparent affinity is reduced because only a small fraction of the peptide molecules are in a nativelike conformation at any time. This model assumes that the antibody binds only those peptide molecules that are in the native conformation. Since the concentration of these is lower than the total peptide concentration by a factor that corresponds to the conformational equilibrium constant of the peptide, the apparent affinity is also lower by this factor. This model is analogous to an allosteric model. A third, intermediate hypothesis would suggest that initial binding of the peptide in a nonnative conformation occurs with submaximal complementarity and is followed by an intramolecular conformational change in the peptide to achieve energy minimization by assuming a nativelike conformation. This third hypothesis corresponds to an induced fit model. The loss of affinity is due to the energy required to change the conformation of the peptide, which in turn corresponds to the conformational equilibrium constant in the second hypothesis. To some extent these models could be distinguished kinetically, since the first hypothesis predicts a faster "on" rate and a faster "off" rate than does the second hypothesis (71).

Although not the only way to explain the data, the second hypothesis is useful because it provides a method to estimate the conformational equilibria of proteins and peptides (42,72). The method assumes the second hypothesis, which can be expressed as follows:



(1)

where A is antibody, P_n is native peptide, and P_r is random conformation peptide, so that

$$K_{apparent} = K_{conf}K_{native} \quad (2)$$

Thus the ratio of the apparent association constant for peptide to the measured association constant for the native molecule should give the conformational equilibrium constant of the peptide. Note the implicit assumption that the total peptide concentration can be approximated by $[P_r]$. This will generally be true, since most peptide fragments of proteins demonstrate little native conformation; that is, $K_{conf} = [P_n]/[P_r]$ is much less than 1. Also note that if the first hypothesis (or third) occurs to some extent, this method will overestimate K_{conf}. On the other hand, if the affinity for the peptide is lower because it lacks some of the contact residues of the determinant, this method will underestimate K_{conf} (by assuming that all the affinity difference is due to conformation). To some extent, the two errors may partially cancel out. When this method was used to determine the K_{conf} for a peptide staphylococcal nuclease, a value of 2×10^{-4} was obtained (42). Similarly, when antibodies raised to a peptide fragment were used, it was possible to estimate the fraction of time the native nuclease spends in nonnative conformations (72). In this case, the K_{conf} was found to be about 3000-fold in favor of the native conformation.

Antipeptide Antibodies that Bind to Native Proteins at a Specific Site

In light of the conformational differences between native proteins and peptides and the observed K_{conf} effects shown by antibodies to native proteins when tested on the corresponding peptides, it was somewhat surprising to find that antibodies to synthetic peptides show extensive cross-reactions with native proteins (73,74). These two types of cross-reactions can be thought of as working in opposite directions: the binding of antiprotein antibodies to the peptide is inefficient, while the binding of antipeptide antibodies to the protein is quite efficient and commonly observed. This finding is quite useful, since automated solid-phase peptide synthesis has become readily available. This has been particularly useful in three areas: exploitation of protein sequences deduced by recombinant DNA methods, preparation of site specific antibodies, and the attempt to focus the immune response on a single protein site that is biologically important but may not be particularly immunogenic. This section focuses on the explanation of the cross-reaction, uses of the cross-reaction, and the potential limitations with regard to immunogenicity.

The basic assumption is that antibodies raised against peptides in an unfolded structure will bind the corresponding site on proteins folded into the native structure (74). This is not immediately obvious, since antibody binding to antigen is the direct result of the antigen fitting into the binding site. Affinity is the direct consequence of "goodness of fit" between antibody and antigen, while antibody specificity is due to the inability of other antigens to occupy the same site. How then can the antipeptide antibodies overcome the effect of K_{conf} and still bind native proteins with good affinity and specificity? The whole process depends on the antibody-binding site forming a three-dimensional space and the antigen filling it in an energetically favorable way.

Since the peptides are randomly folded, they rarely occupy the native conformation, so they are not likely to elicit antibodies against a conformation they do not maintain. If the antibodies are specific for a denatured structure, then, like the myoglobin molecules that were denatured to apomyoglobin by antibody binding (43), the cross-reaction may depend on the native protein's ability to assume different conformational states. If the native protein is quite rigid, then the possibility of its assuming a random conformation is quite small; but if it is a flexible three-dimensional spring, then local unfolding and refolding may occur all the time. Local unfolding of protein segments may permit the immunologic cross-reaction with antipeptide antibodies, since a flexible segment could assume many of the same conformations as the randomly folded peptide (74).

In contrast, the proteins' ability to crystallize (a feature that allows the study of their structure by x-ray crystallography) has long been taken as evidence of protein rigidity (75). In addition, the existence of discrete functional states of allosteric enzymes (76) provides additional evidence of stable structural states of a protein. Finally, the fact that antibodies can distinguish native from denatured forms of intact proteins is well known for proteins such as myoglobin (43).

However, protein crystals are a somewhat artificial situation, since the formation of the crystal lattice imposes order on the components, each of which occupies a local energy minimum at the expense of considerable loss of randomness (entropy). Thus the crystal structure may have artificial rigidity that exceeds the actual rigidity of protein molecules in solution. On the contrary, we may attribute some of the considerable difficulty in crystallizing proteins to disorder within the native conformation. Second, allosterism may be explained by two distinct conformations that are discrete without being particularly rigid. Finally, the ability to generate antiprotein antibodies that are conformation specific does not rule out

the existence of antipeptide antibodies that are not. All antibodies are probably specific for some conformation of the antigen, but this need not be the crystallographic native conformation in order to achieve a significant affinity for those proteins or protein segments that have a "loose" native conformation.

Antipeptide antibodies have proved to be very powerful reagents when combined with recombinant DNA methods of gene sequencing (74,77). From the DNA sequence, the protein sequence is predicted. A synthetic peptide is constructed, coupled to a suitable carrier molecule, and used to immunize animals. The resulting polyclonal antibodies can be detected with a peptide-coated ELISA plate (see Chapter 12). They are used to immunoprecipitate the native protein from a ^{35}S-labeled cell lysate and thus confirm expression of the gene product in these cells. The antipeptide antibodies can also be used to isolate the previously unidentified gene product of a new gene. The site specific antibodies are also useful in detecting post-translational processing, since they bind all precursors and products that contain the site. In addition, since the antibodies bind only to the site corresponding to the peptide, they are useful in probing structure–function relationships. They can be used to block the binding of a substrate to an enzyme or the binding of a virus to its cellular receptor.

Immunogenicity of Proteins and Peptides

Up to this point, we have considered the ability of antibodies to react with proteins or peptides as antigens. However, immunogenicity refers to the ability of these compounds to elicit antibodies following immunization. In principle, nearly the entire surface of a globular protein should be able to elicit antibodies, particularly when we allow for both topographic and segmental specific antibodies (40). However, several factors limit the immunogenicity of different regions of proteins, and these have been divided into those that are intrinsic to protein structure itself versus those extrinsic to the antigen that are related to the responder and vary from one animal or species to another (41). In addition, we consider the special case of peptide immunogenicity as it applies to vaccine development.

Studies of intrinsic factors began by immunizing animals with native proteins and analyzing the antibodies that resulted. With polyclonal antisera, it is necessary to compare the relative amounts of antibodies directed at each site or class of sites: immunodominant sites are the ones that elicit the most antibodies. Monoclonal antibodies advanced our ability to study the immunogenicity of discrete sites. The features of protein structure that have been suggested to explain the results include surface accessibility of the site, hydrophilicity, flexibility, and proximity to a site recognized by helper T cells.

When the x-ray crystallographic structure and antigenic structure are known for the same protein, it is not surprising to find that a series of monoclonal antibodies binding to a molecule such as influenza neuraminidase choose an overlapping pattern of sites at the exposed head of the protein (78). The stalk of neuraminidase was not immunogenic, apparently because it was almost entirely covered by carbohydrate.

Beyond such things as carbohydrate, which may sterically interfere with antibody binding to protein, accessibility on the surface is clearly a *sine qua non* for an antigenic determinant to be bound by an antibody specific for the native conformation, without any requirement for unfolding of the structure (41). Several measures of such accessibility have been suggested. All these require knowledge of the x-ray crystallographic three-dimensional structure. Some have measured accessibility to solvent by rolling a sphere with the radius of a water molecule over the surface of a protein (79,80). Others have suggested that accessibility to water is not the best measure of accessibility to antibody and have demonstrated a better correlation by rolling a sphere with the radius of an antibody-combining domain (81). Another approach to predicting antigenic sites on the basis of accessibility is to examine the degree of protrusion from the surface of the protein (69). This was done by modeling the body of the protein as an ellipsoid and examining which amino acid residues remain outside ellipsoids of increasing dimensions. The most protruding residues were found to be part of antigenic sites bound by antibodies, but usually these sites had been identified by using short synthetic peptides and so were segmental in nature. As noted above, for an antigenic site to be contained completely within a single continuous segment of protein sequence, the site is likely to have to protrude from the surface, as otherwise residues from other parts of the sequence would fall within the area contacting the antibody (39).

Because the three-dimensional structure of most proteins is not known, other ways of predicting surface exposure have been proposed for the vast majority of antigens. For example, hydrophilic sites tend to be found on the water-exposed surface of proteins and could be favored targets for antibodies. Thus hydrophilicity has been proposed as a second indication of immunogenicity (82–84). This model has been used to analyze 12 proteins with known antigenic sites: the most hydrophilic site of each protein was indeed one of the antigenic sites. However, among the limitations are the facts that a significant fraction of surface residues can be nonpolar (79,80) and that several important examples of hydrophobic and aromatic amino acids involved in the antigenic sites are known (33,64,85,86). Specificity of antibody binding likely depends on the complementarity of surfaces for hydrogen bonding and polar bonding as well as van der Waals contacts (87), while hydrophobic interactions and

the exclusion of water from the interacting surfaces of proteins may contribute a large but nonspecific component to the energy of binding (87).

A third factor suggested to play a role in immunogenicity of protein epitopes is mobility. Measurement of mobility in the native protein is largely dependent on the availability of a high-resolution crystal structure, so its applicability is limited to only a small subset of proteins. Furthermore, it has been studied only for antibodies specific for segmental antigenic sites; therefore it may not apply to the large fraction of antibodies to assembled topographic sites. Studies of mobility have taken two directions. The case of antipeptide antibodies has already been discussed, in which antibodies made to peptides corresponding to more mobile segments of the native protein were more likely to bind to the native protein (74,88). This is not considered just a consequence of the fact that more mobile segments are likely to be those on the surface and therefore more exposed, because in the case of myohemerythrin (which was used as a model), two regions of the native protein that were equally exposed but less mobile did not bind nearly as well to the corresponding antipeptide antibodies (89). However, as is clear from the earlier discussion, this result applies to antibodies made against short peptides and therefore is not directly relevant to immunogenicity of parts of the native protein. Rather, it concerns the cross-reactivity of antipeptide antibodies with the native protein and therefore is of considerable practical importance for the purposes outlined in the section on antipeptide antibodies.

Studies in the other direction—that is, of antibodies raised against native proteins—would be by definition more relevant to the question of immunogenicity of parts of the native protein. Westhof et al. (90) used a series of hexapeptides to determine the specificity of antibodies raised against native tobacco mosaic virus protein and found that six of the seven peptides that bound antibodies to native protein corresponded to peaks of high mobility in the native protein. The correlation was better than could be accounted for just by accessibility, because three peptides that corresponded to exposed regions of only average mobility did not bind antibodies to the native protein. However, when longer peptides—on the order of 20 amino acid residues—were used as probes, it was found that antibodies were present in the same antisera that bound to less mobile regions of the protein (91). They simply had not been detected with the short hexapeptides with less conformational stability. Thus it was not that the more mobile regions were necessarily more immunogenic but rather that antibodies to these were more easily detected with short peptides as probes. A similar good correlation of antigenic sites with mobile regions of the native protein in the case of myoglobin (90) may also be attributed to the fact that seven of the nine sites were defined with short peptides of six to eight residues (55). Again, this result becomes a statement about cross-reactivity between peptides and native protein rather than about the immunogenicity of the native protein. For recent reviews, see Van Regenmortel (92) and Getzoff et al. (32).

To address the role of mobility in immunogenicity, an attempt was made to quantitate the relative fraction of antibodies specific for different sites on the antigen myohemerythrin (93). The premise was that, although the entire surface of the protein may be immunogenic, certain regions may elicit significantly more antibodies than others and therefore may be considered immunodominant or at least more immunogenic. Since this study was done with short synthetic peptides from 6 to 14 residues long based on the protein sequence, it was limited to the subset of antibodies specific for segmental antigenic sites. Among these, it was clear that the most immunogenic sites were in regions of the surface that were most mobile, convex in shape, and often of negative electrostatic potential. The role of these parameters has been covered in a recent review (32).

These results have important practical and theoretical implications. First, to use peptides to fractionate anti-protein antisera by affinity chromatography, peptides corresponding to more mobile segments of the native protein should be chosen when possible. If the crystal structure is not known, it may be possible to use peptides from amino or carboxyl termini or from exon–intron boundaries, as these are more likely to be mobile (88). Second, these results may explain how a large but finite repertoire of antibody-producing B cells can respond to any antigen in nature or even artificial antigens never encountered in nature. Protein segments that are more flexible may be able to bind by induced fit in an antibody-combining site that is not perfectly complementary to the average native structure (32,41). Indeed, evidence from the crystal structure of antigen–antibody complexes (94–96) suggests that mobility in the antibody-combining site as well as in the antigen may allow both reactants to adopt more complementary conformations on binding to each other, that is, a two-way induced fit. A very nice example comes from the study of antibodies to myohemerythrin (95), in which the data suggested that initial binding of exposed side chains of the antigen to the antibody promoted local displacements that allowed exposure and binding of other, previously buried residues. The role of these critical amino acid side chains that are buried in the native crystal structure appeared to be one of contact residues with the antibody-combining site rather than one of stabilization of a particular conformation. The only way this could occur would be for such residues to become exposed during the course of an induced fit conformational change in the antigen (32,95). In a second very clear example of induced fit, the contribution of antibody mobility to peptide binding was demonstrated for a monoclonal antibody to peptide 75–110 of influenza

hemagglutinin, which was crystallized with or without peptide in the binding site and analyzed by x-ray crystallography for evidence of an induced fit (96). Despite flexibility of the peptide, the antibody-binding site probably could not accommodate the peptide without a conformational change in the third complementarity determining region (CDR3) of the heavy chain, in which an asparagine residue of the antibody was rotated out of the way to allow a tyrosine residue of the peptide to fit in the binding pocket of the antibody (96).

With regard to host-limited factors, immunogenicity is certainly limited by self-tolerance. Thus the repertoire of potential antigenic sites on mammalian protein antigens such as myoglobin or cytochrome *c* can be thought of as greatly simplified by the sharing of numerous amino acids with the endogenous myoglobin or cytochrome of the host. For mouse, guanaco, or horse cytochrome *c* injected into rabbits, each of the differences between the immunogen and rabbit cytochrome *c* is seen as an immunogenic site on a background of immunologically silent residues (40,65,97). In another example, antibodies to beef myoglobin were made in various species (98). Rabbit and dog antibodies bound almost equally well to beef or sheep myoglobin. However, sheep antibodies bound beef but not sheep myoglobin, even though these two myoglobins differ by just six amino acids. Thus the sheep immune system was able to screen out those clones that would be autoreactive with sheep myoglobin.

Ir genes of the host also play an important role in regulating the ability of an individual to make antibodies to a specific antigen (99). These antigen specific immunoregulatory genes are among the MHC genes that code for transplantation antigens. Structural mutations, gene transfer experiments, and biochemical studies (99) all indicate that *Ir* genes are actually the structural genes for MHC antigens. The mechanism of action of the MHC antigens works through their effect on helper T cells (described later). Briefly, T cells can respond to a protein antigen only when a fragment of the protein is bound to an MHC antigen, forming a compound antigen. T cells must be activated before they can help B cells respond to antigen by both expanding the appropriate clones of antigen specific B cells and differentiating the expanded clones into antibody-secreting plasma cells. There appear to be constraints on which B cells a T cell of a given specificity can help (100,101), a process called T–B reciprocity (102). Thus if *Ir* genes control helper T cell specificity, they will in turn limit which B cells are activated and thus which antibodies are made.

The immunogenicity of peptide antigens is also limited by intrinsic and extrinsic factors. Intrinsic features such as hydrophilicity and surface accessibility are not a problem, until we consider the cross-reaction of the elicited antibodies on the native protein. Bigger problems are the host-related factors extrinsic to the structure of the peptide. With less structure to go on, each small peptide must presumably contain some nonself structural feature in order to overcome self-tolerance. In addition, the same peptide must contain antigenic sites that can be recognized by helper T cells as well as by B cells. When no T cell site is present, three approaches may be helpful: graft on a T cell site; couple the peptide to a carrier protein; or overcome T cell nonresponsiveness to the available structure with various immunologic agents, such as interleukin 2.

An example of a biologically relevant but poorly immunogenic peptide is the asparagine–alanine–asparagine–proline (NANP) repeat unit of the circumsporozoite (CS) protein of malaria sporozoites. Studies with malaria parasites have shown that infection by the sporozoite form produced in the mosquito can be blocked by prior immunization with irradiated, killed sporozoites (103). A monoclonal antibody to CS protein can mimic the effect in murine malaria, and this antibody is specific for the repeat unit of the CS protein (104). Thus it would be desirable to make a malaria vaccine of the repeat unit of *P. falciparum* $(NANP)_n$. However, only mice of one MHC type ($H\text{-}2^b$) of all mouse strains tested were able to respond to $(NANP)_n$ (105,106). One approach to overcome this limitation is to couple $(NANP)_n$ to a site recognizable by T cells, perhaps a carrier protein such as tetanus toxoid (107). In human trials, this conjugate was weakly immunogenic and only partially protective. Moreover, as helper T cells produced by this approach are specific for the unrelated carrier, a secondary or memory response would not be expected to be elicited by the pathogen itself.

Another choice might be to identify a T cell site on the CS protein itself and couple the two synthetic peptides together to make one complete immunogen. The result with one such site, called Th2R, was to increase the range of responding mouse MHC types by one, to include $H\text{-}2^k$ as well as $H\text{-}2^b$ (108). This approach has the potential advantage of inducing a state of immunity that could be boosted by natural exposure to the sporozoite antigen. Since CS specific T and B cells are both elicited by the vaccine, natural exposure to the antigen could help maintain the level of immunity during the entire period of exposure.

Another strategy to improve the immunogenicity of peptide vaccines is to stimulate the T and B cell responses artificially by adding interleukin 2 to the vaccine. Results with myoglobin indicate that genetic nonresponsiveness can be overcome by appropriate doses of interleukin 2 (109). The same effect was found for peptides derived from malaria proteins (110; K. Akaji, D. T. Liu, and I. J. Berkower, *unpublished results*). It is not yet clear whether this effect is based on immunizing T cells or whether low T cell responsiveness is overcome by a direct effect on B cells. In the former case, active immunity could result once specific T and B

cell clones are expanded, even in individuals who would otherwise be nonresponders.

One of the most important possible uses of peptide antigens is as synthetic vaccines. However, even though it is possible to elicit with synthetic peptides anti-influenza antibodies to nearly every part of the influenza hemagglutinin (73), antibodies that neutralize viral infectivity have not been elicited by immunization with synthetic peptides. This may reflect the fact that antibody binding by itself often does not result in virus inactivation. Viral inactivation occurs only when antibody interferes with one of the steps in the life cycle of the virus, including binding to its cell surface receptor, internalization, and virus uncoating within the cell. Apparently, antibodies can bind to most of the exposed surface of the virus without affecting these functions. Only those antibodies that bind to certain "neutralizing" sites can inactivate the virus. In addition, as in the case of the VP1 coat protein of poliovirus, certain neutralizing sites are found only on the native protein and not on the heat-denatured protein (111). Thus not only the site but also the conformation that is bound by the antibodies may be important for the antibody to inactivate the virus. These sites may often be assembled topographic sites not mimicked by peptide segments of the sequence. Perhaps binding of an antibody to such an assembled site can alter the relative positions of the component subsites so as to induce an allosteric neutralizing effect. Alternatively, antibodies to such an assembled site may prevent a conformational change necessary for activity of the viral protein.

One method of mapping neutralizing sites is based on the use of neutralizing monoclonal antibodies. The virus is grown in the presence of neutralizing concentrations of the monoclonal antibody, and virus mutants are selected for the ability to overcome antibody inhibition. These are sequenced, revealing the mutation that permits "escape" by altering the antigenic site for that antibody. This method has been used to map the neutralizing sites of influenza hemagglutinin (112) as well as poliovirus capsid protein VP1 (113). The influenza escaping mutations are clustered to form an assembled topographic site, with mutations distant from each other in the primary sequence of hemagglutinin but brought together by the three-dimensional folding of the native protein. At first, it was thought that neutralization was the result of steric hindrance of the hemagglutinin binding site for the cell surface receptor of the virus (114). However, similar work with poliovirus reveals that neutralizing antibodies that bind to assembled topographic sites may inactivate the virus at less than stoichiometric amounts, when at least half of the sites are unbound by antibody (115). The neutralizing antibodies all cause a conformational change in the virus, which is reflected in a change in the isoelectric point of the particles from pH 7 to pH 4 (113,116). Antibodies that bind without neutralizing do not cause this shift. Thus an alternative explanation for the mechanism of antibody-mediated neutralization is the triggering of the virus to self destruct. Perhaps the reason that neutralizing sites are clustered near receptor-binding sites is that occupation of such sites by antibody mimics events normally caused by binding to the cellular receptor, causing the virus to prematurely trigger its cell entry mechanisms. However, in order to transmit a physiologic signal, the antibody may need to bind viral capsid proteins in the native conformation (especially assembled topographic sites), which antipeptide antibodies may fail to do. Antibodies of this specificity are similar to the viral receptors on the cell surface, some of which have been cloned and expressed without their transmembrane sequences as soluble proteins. The soluble recombinant receptors for poliovirus (117) and HIV-1 (118–120) exhibit high-affinity binding to the virus and potent neutralizing activity *in vitro*. The HIV-1 receptor, CD4, has been combined with the human immunoglobulin heavy chain in a hybrid protein CD4–Ig (121), which spontaneously assembles into dimers and resembles a monoclonal antibody, in which the binding site is the same as the receptor-binding site for HIV-1. In these recombinant constructs, high-affinity binding depends on the native conformation of the viral envelope glycoprotein gp120.

For HIV-1, two types of neutralizing antibodies have been identified. The first type binds a continuous or segmental determinant, the "V3 loop" sequence between amino acids 296 and 331 of gp120 (122). Antipeptide antibodies against this site can neutralize the virus (123). However, because this site is located in a highly variable region of the envelope, these antibodies tend to neutralize a limited range of viral variants. A second type of neutralizing antibody binds at or near the CD4 receptor-binding site of gp120 (124–126). These neutralizing antibodies, which are commonly found in the sera of infected patients, are specific for a broad range of HIV-1 variants, possibly due to conserved sequences around the CD4 binding site (127). Since the shared neutralizing determinant is an assembled topographic site, dependent on the native conformation of the protein (128), a prospective gp120 vaccine would need to be in the native conformation to be able to elicit these antibodies.

ANTIGENIC DETERMINANTS RECOGNIZED BY T CELLS

Mapping Antigenic Structures

Studies of T cell specificity for antigen were motivated by the fact that the immune response to protein antigens is regulated at the T cell level. A hapten, not immunogenic by itself, will elicit antibodies only when coupled to a protein that elicits a T cell response in that animal. This ability of the protein component of the conjugate to

confer immunogenicity on the hapten has been termed the "carrier effect." Recognition of the carrier by specific helper T cells induces the B cells to make antibodies. Thus the factors contributing to a good T cell response appear to control the B cell response as well.

"Nonresponder" animals display an antigen specific failure to respond to a protein antigen, both for T cells and antibody responses. The "high responder" phenotype for each antigen is a genetically inheritable, usually dominant trait. Using inbred strains of mice, the genes controlling the immune response were found to be tightly linked to the major histocompatibility complex (MHC) of genes (99,129). MHC-linked immune responsiveness has been shown to depend on the T cell recognition of antigen in association with MHC antigens of the antigen-presenting cell (APC) (discussed later; see also Chapter 17). The recognition of antigen in association with MHC molecules of the B cell is necessary for carrier specific T cells to expand and provide helper signals to B cells, which results in clonal expansion and maturation of the B cells into antibody-producing cells.

In contrast to the range of antigens recognized by antibodies, the repertoire recognized by helper and cytotoxic T cells appears to be limited largely to protein and peptide antigens, although exceptions such as the small molecule tyrosine–azobenzene arsonate (130) exist. This limitation is not understood but may relate to the requirement for binding of antigens within a groove of the MHC molecule (see below).

If the antigenic determinants on proteins recognized by T cells could be identified, then it would be possible to better understand immunogenicity, to explain immune-response genes, and perhaps even to enhance the antibody response to one part of a biologically relevant antigen by altering the T cell response for another part of the antigen.

Polyclonal T Cell Response

Significant progress in understanding T cell specificity was made possible by focusing on T cell proliferation *in vitro*. Proliferation of antigen specific cells in culture mimics the positive selection via clonal expansion of antigen specific clones that occurs *in vivo*. The proliferative response depends on only two cells: the antigen specific T cell and an APC, usually a macrophage, dendritic cell, or B cell. The growth of T cells in culture is measured in terms of the incorporation of [^{3}H]thymidine into newly formed DNA. Under appropriate conditions, thymidine incorporation increases with antigen concentration. This assay permits the substitution of different APCs and is highly useful in defining the MHC and antigen-processing requirements of the APCs.

Using primarily this assay, several different approaches have been taken to mapping T cell epitopes. First, naturally occurring variants of the native protein, such as homologous proteins from different species with known amino acid substitutions, have been used to identify positions in the protein sequence at which substitutions could affect T cell cross-reactivity. Usually, this has led to correct localization of the antigenic site in the protein (131–133), but the possibility of long-range effects on antigen processing must be kept in mind (see the section entitled Antigen Processing). Also this approach is limited in that it can focus on the correct region of the molecule but cannot define the boundaries of the site or identify all the critical residues because it is limited to testing those positions in the sequence at which amino acid substitutions occur in natural variants. Site-directed mutagenesis may therefore expand the capabilities of this approach. A second approach is to use short peptide segments of the protein sequence, taking advantage of the fact that T cells specific for soluble protein antigens appear to see only segmental antigenic sites, not assembled topographic ones (99,134–138; see the section entitled Antigen Processing). These may be produced by chemical or enzymatic cleavage of the natural protein (136–143), solid-phase peptide synthesis (143–146), or recombinant DNA methodology using expression of cloned genes or gene fragments (147). In the case of class I MHC molecule-restricted cytotoxic T cells, viral gene deletion mutants expressing only part of the gene product have also been used (148).

For example, the T cell response to sperm whale myoglobin was analyzed in mice of two high responder MHC types, H-2^s and H-2^d. When H-2^s mice were immunized with sperm whale myoglobin, the T cells responded in culture to sperm whale myoglobin and about half of the 12 additional mammalian myoglobins tested (132), but they did not respond to several other whale myoglobins or to horse myoglobin (Table 5). Conversely, when mice were immunized to horse myoglobin, they responded to horse myoglobin, and the pattern of myoglobin cross-reactivity was the reciprocal of that seen in T cells from animals immunized with sperm whale myoglobin: each myoglobin that stimulated these T cells did not stimulate sperm-whale-immune T cells and vice versa. The response to the cross-stimulatory myoglobins was as strong as to the myoglobin used to immunize the mice. This suggested that a few shared amino acid residues formed an immunodominant epitope that was essential for T cell activation and that most substitutions had no effect on the dominant epitope. Comparing those amino acid residues that were conserved in the stimulatory myoglobins with those that were substituted in the nonstimulatory myoglobins revealed that substitutions at a single residue could explain the pattern observed. All myoglobins that cross-stimulated sperm-whale-immune T cells had Glu at position 109, while all that cross-stimulated horse-immune T cells had Asp at 109. No member of one group could stimulate T cells from donors immu-

TABLE 5. *Proliferation of B10.S T cells immune to myoglobin*

Stimulating myoglobin	Residue number	T cells immune to: Equine	T cells immune to: Sperm whale
	1 1 2 2 2 3 3 4 6 6 7 / 1 4 9 2 5 1 7 8 4 5 5 6 7 4 / G D Q N G I E V T G K T V G	1 / 0 / 9 / D	1 1 1 1 1 / 1 2 3 4 5 / 8 2 2 0 1 / K N T N F		
1. Equine		–		11,580	72
2. Bovine	A V N T	–	S	11,305	N.D.[a]
3. Sei whale	V L A V D I K N T	–	R D N K	20,922	1,205
4. Minke whale	V L A V D I K N T	–	R E N K	13,604	3,694
5. Goosebeaked whale	E L H A L I K H T	–	R D K	10,373	1,500[b]
6. Dog	I L K N N T	–	D K	6,863	610[b]
7. California sea lion	L L K K T	E	D K	683	6,869
8. Harbor seal	L L K S N T	E	E K	3,132	9,592
9. Killer whale	L L D I K N T A	E	R E N K	2,568	16,745
10. Dall porpoise	E L L D K N T	E	R E N K	1,759	7,653
11. Sperm whale	V E L H A V D I K S R V T A	E	R D N K Y	3,500	18,593
Medium control				1,164	611

[a] N.D., not done.
[b] Normalized results from a second experiment.

nized with a myoglobin of the other group. This suggested that an immunodominant epitope recognized by T cells was centered on position 109, regardless of which amino acid was substituted. But the T cells elicited by a myoglobin of either group could readily distinguish between Asp or Glu at this position. Similar results were obtained with cytochrome *c*, where the predominant site recognized by T cells was localized with sequence variants to the region around residue 100 at the carboxyl end of cytochrome (131). Furthermore, the response to cytochrome *c* peptide 81 to 104 was as great as the response to the whole molecule. This indicated that a 24 amino acid peptide contained an entire antigenic site recognized by T cells. Subsequent studies with synthetic peptides indicated that the T cells could distinguish between peptides with Lys or Gln at position 99, although both were immunogenic with the same MHC molecule (149–151). This residue determined T cell memory and specificity and so presumably was interacting with the T cell receptor. A similar conclusion could be drawn for residue 109 of myoglobin. The ultimate use of synthetic peptides to analyze the segmental sites of a protein that are recognized by T cells was to synthesize a complete set of peptides, each staggered by just one amino acid from the previous peptide, corresponding to the entire sequence of hen egg lysozyme, HEL (152). Around each immunodominant site, a cluster of several stimulatory peptides was found. The minimum "core" sequence consisted of just those residues shared by all antigenic peptides within a cluster, while the full extent of sequences spanning all stimulatory peptides within the same cluster defined the "determinant envelope." These two ways of identifying an antigenic site differ, and one interpretation is that each core sequence corresponds to an MHC binding site, while the determinant envelope includes the many ways for T cells to recognize the same peptide bound to the MHC.

In each case, the polyclonal T cell response could be mapped to a single predominant antigenic site. These results are consistent with the idea that each protein antigen has a limited number of immunodominant sites (possibly one) recognized by T cells in association with MHC molecules of the high responder type. If none of the antigenic sites could associate with MHC molecules on the APCs, then the strain would be a low responder to that antigen. The antigen would be unable to stimulate T cells, resulting in little or no immunogenicity.

Monoclonal T Cells

Further progress in mapping T cell sites depended on the analysis of cloned T cell lines. These were either antigen specific T cell lines made by the method of Komoto and Fathman (153) or T cell hybridomas made by the method of Kappler and co-workers (154). In the former method, T cells are allowed to proliferate in response to antigen, rested, and then restimulated by an additional round of antigen. After each stimulation, the blasts are selected by centrifugation on Ficoll to enrich further for antigen specific T cells. They are then cloned by limiting dilution and grown from a single cell. The antigen specificity of the line is confirmed in the standard proliferation assay using antigen and APCs. In the second method, enriched populations of antigen specific T cells are fused with a drug-sensitive T cell tumor, and the fused cells are selected for their ability to grow in the presence of the drug. Then the antigen specificity of each

fused cell line must be determined. The key to determining this in a tumor line is that antigen specific stimulation of a T cell hybridoma results in release of interleukin 2 even though proliferation is constitutive. T cells produced by either method are useful in defining epitopes, measuring their MHC associations, and studying antigen-processing requirements.

Monoclonal T cells may be useful in identifying which of the many proteins from a pathogen are important for T cell responses. For instance, Young and Lamb (155) have developed a way to screen proteins separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose for stimulation of T cell clones and have used this to identify antigens of *Mycobacterium tuberculosis* (156). Mustafa et al. (157) have even used T cell clones to screen recombinant DNA expression libraries to identify relevant antigens of *M. leprae*.

Monoclonality is important in ascertaining whether different specificities or functions are properties of the same T cell. For example, each myoglobin specific clone was tested for both antigen specificity and MHC restriction. Six T cell clones were found to be specific for the predominant Glu 109 site, and all six recognized the antigen only in association with I-A^d. Nine additional T cell clones were specific for a second epitope centered on Lys 140 and were restricted to a different MHC antigen, I-E^d. Thus each T cell's specificity involved both a restriction to an antigenic epitope and a restriction to a particular MHC molecule of the APC (158).

Precise mapping of antigenic sites recognized by T cells was made possible by the fact that T cells would respond to peptide fragments of the antigen when they contain a complete antigenic determinant. First, a critical amino acid residue, such as Glu 109 or Lys 140, was found by comparing the sequences of stimulatory and nonstimulatory myoglobin variants and large CNBr cleavage fragments (159), and then a series of truncated peptides containing the critical residue was synthesized with different overlapping lengths at either end (143,146). Because solid-phase peptide synthesis starts from a fixed carboxyl end and proceeds toward the amino end, it is easy to stop the synthesis at various positions past the critical residue and remove some peptide, then proceed with the synthesis. In this way, a nested series of peptides that vary in length at the amino end can quickly be prepared. Each peptide is then tested as the antigen in the proliferation assay. In this way, it was found that two of the Glu 109 specific T cell clones responded to synthetic peptides 102 to 118 and 106 to 118 but not to peptide 109 to 118 (146). One clone responded to peptide 108 to 118, while the other did not. Thus the amino end of the peptide recognized by one clone was Ser 108, while the other clone required Phe 106 and/or Ile 107. Similar fine specificity differences have been observed with T cell clones specific for the peptides 52 to 61 and 74 to 96 of hen egg lysozyme (140,160,161), the peptide 323 to 339 of chicken ovalbumin (141), and the peptide 81 to 104 of pigeon cytochrome *c* (145): the epitopes recognized by several T cell clones overlap but are distinct. In addition, a second T cell determinant in myoglobin located around Lys 140 was found. Nine T cell lines specific for this determinant responded to the cyanogen bromide cleavage fragment 132 to 153 (158). Further studies with a nested series of synthetic peptides (peptide 135 to 146 versus 136 to 146 versus 137 to 146, etc., in Fig. 6) showed that the stimulatory sequence is contained in peptide 136 to 146, while additional studies with peptides trimmed at the carboxyl end with carboxypeptidases B and A showed that Lys 145 is necessary, but Tyr 146 is not, although it contributes to antigenic potency (143). What these studies demonstrated about epitopes recognized by T cells is that they are segmental determinants, contained on synthetic peptides consisting of no more than about 12 amino acids. Within this size, they must contain all the information necessary to survive processing within the APC, associate with the MHC antigen, and bind to the T cell receptor.

These findings can be generalized to characterize a large number of epitopes recognized by T cells from a number of protein antigens (Table 6). In each case, the entire site is contained on a short peptide antigen. Perhaps unexpectedly, MHC class I-restricted antigens also follow this rule (174). This even applies to viral glycopro-

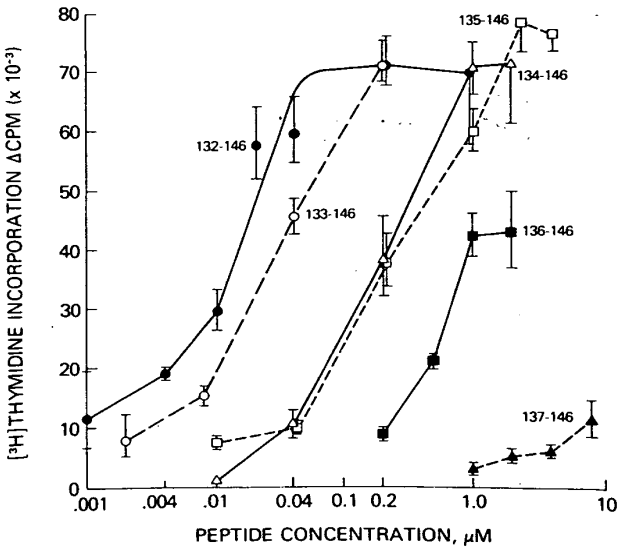


FIG. 6. Proliferative response of T cell clone 14.5 to a series of synthetic peptides that correspond to the sequence 132 to 146 of sperm whale myoglobin. Comparison of the response to peptides that differ by a single amino acid residue at the amino end shows that Lys 133 is important for potency but is not critical for antigenicity; Glu 136 is critical for antigenicity. Background proliferation was 5561 cpm. (From ref. 143, with permission.)

TABLE 6. *Examples of immunodominant T cell epitopes recognized in association with class II MHC molecules*[a]

Protein	T cell antigenic sites (reference number)	Amphipathic segments
Sperm whale myoglobin	69–78 (136)	64–78
	102–118 (146)	99–117
	132–145 (143)	128–145
Pigeon cytochrome *c*	93–104 (145)	92–103
Influenza	109–119 (162)	97–120
Hemagglutinin	130–140 (163)	—
A/PR/8/34 Mt.S.	302–313 (163,164)	291–314
Pork insulin	(B)5–16 (144)	4–16
	(A)4–14 (165)	1–21
Chicken lysozyme	46–61 (161)	—
	74–86 (160)	72–86
	81–96 (160)	86–102
	109–119 (133)	—
Chicken ovalbumin	323–339 (141)	329–346
Hepatitis B virus pre-S	120–132 (166)	121–135
Foot and mouth virus VP1	141–160 (167)	148–165
Beef cytochrome *c*	11–25 (168)	9–29
	66–80 (169)	58–78
Hepatitis B virus	38–52 (170)	36–49
Major surface	95–109 (170)	—
Antigen	140–154 (170)	—
λ Repressor protein CI	12–26 (171)	8–25
Rabies virus-spike glycoprotein precursor	32–44 (172)	29–46

[a] Adapted from ref. 173.

teins, such as influenza hemagglutinin, that are normally expressed on the surface of infected cells. Cytolytic T cells also recognize these antigens after antigen processing (175).

Antigen Processing

Processing Antigen for T Cells Restricted to Class II MHC Molecules

Unlike B cells, T cell recognition of antigen depends on the function of another cell, the APC (176). Its functions are shown in Fig. 7: (a) it takes up the antigen by phagocytosis, pinocytosis, or, in some cases, receptor-mediated endocytosis; (b) it partially degrades foreign antigens into discrete antigenic fragments that can be recognized by T cells; and (c) it displays these antigenic fragments on the cell surface in association with MHC molecules. Antigen presentation provides a way of focusing antigen on the APC surface in association with MHC molecules in a form that provides an effective antigenic stimulus to T cells.

It has long been known that T cell responses such as delayed hypersensitivity *in vivo* or T cell proliferation *in vitro* to exogenous proteins can be stimulated not only by the native protein but also by denatured protein (134) and fragments of native protein (165). Indeed, this feature, along with the requirement for recognition in association with class II MHC molecules, distinguishes T from B cell responses. In a number of cases, the site recognized by cloned T cells has been located to a discrete synthetic peptide corresponding to a segment of the primary sequence of the protein. Examples include insulin (144,165), cytochrome *c* (145), lysozyme (140,160), and myoglobin (136,143,146). In each case, the stimulatory peptide must contain all the information required for antigen presentation and T cell stimulation. The native protein conformation is lacking, although the most antigenic peptides may or may not be long enough to have significant secondary structure (helicity or β sheet) (177–180). The lack of conformational specificity is not a feature of T cell receptor specificity. Rather, it suggests that something happens to the antigen that destroys conformational differences prior to binding the T cell receptor. One way to accomplish this is via antigen processing, which involves the partial degradation of a protein antigen into peptide fragments (see Fig. 7). The peptides lack the native conformation but of course retain primary sequence. This sequence may serve as the common molecular signal that is recognized by T cells responding to either native or denatured antigen.

That T cells recognize processed antigen was demonstrated by the fact that inhibitors of processing can block antigen presentation. Early experiments by Ziegler and

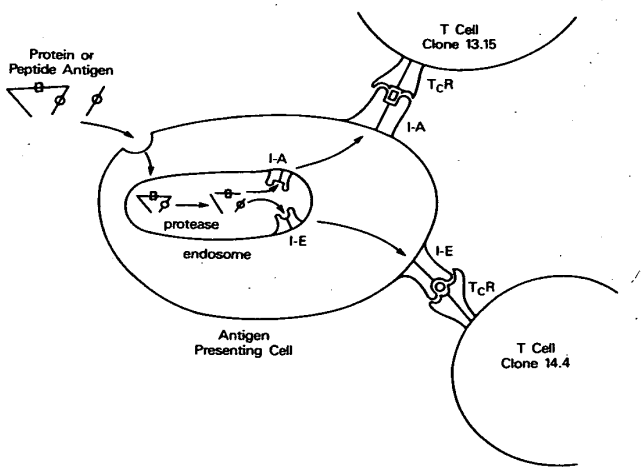


FIG. 7. Steps in antigen presentation by class II MHC molecules. Soluble antigen enters the presenting cell by phagocytosis, pinocytosis, or receptor-mediated endocytosis. It is partially degraded to peptide fragments by acid-dependent endosomal proteases. If antigenic peptides are formed, they will associate with MHC class II molecules (I-A or I-E in the mouse) to form an antigenic complex that is transported to the cell surface. Binding of T cell receptors to the complex triggers the T cell to divide, resulting in clonal expansion of antigen specific T cells.

Unanue (181) showed that processing depends on intracellular degradative endosomes, since drugs like chloroquine and NH_4Cl, which raise endosomal pH and inhibit acid-dependent proteases, could block the process. However, degradation of proteins into peptide fragments allows them to trigger T cells even in the presence of these inhibitors of processing (182). For example, T cell clone 14.5 recognizes the Lys 140 site of myoglobin equally well on the antigenic peptide (residues 132 to 153) as on the native protein (Fig. 8). The difference between these two forms of antigen is brought out by the presence of processing inhibitors. Leupeptin, for example, inhibits lysosomal proteases and blocks the T cell responses to native myoglobin but not to peptide 132 to 153. Thus native myoglobin cannot stimulate T cells without further processing, whereas the peptide requires little or no additional processing (183).

The capability of processed peptides plus class II MHC alone to trigger T cells was shown by Watts et al. (184). They constructed planar membranes containing lipid and purified class II MHC molecules. These membranes could present a peptide fragment of ovalbumin (but not native ovalbumin) to an ovalbumin specific T cell hybridoma. Thus antigen presentation requires only two molecules, and one of these must be partially digested in a prior antigen-processing step. The sufficiency of antigen and MHC was also shown using liposomes (185).

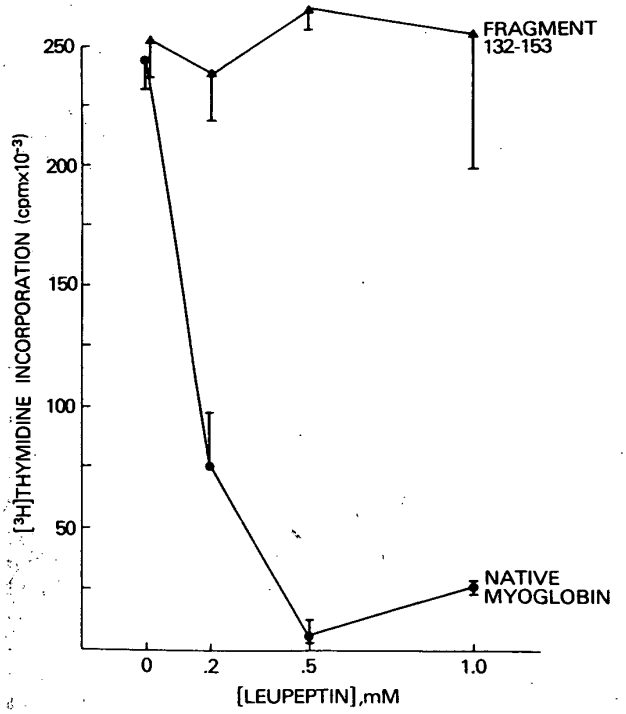


FIG. 8. Inhibition of antigen presentation by the protease inhibitor leupeptin: differential effect on presentation of the same epitope of native myoglobin or peptide 132 to 153 to the same monoclonal T cell population. Splenic cells from nonimmunized B10.D2 mice, as a source of APCs, were incubated with leupeptin at the concentration indicated for 15 min prior to and during exposure to 2 μM native myoglobin or 1 μM peptide fragment, washed, irradiated, and cultured at 400,000 cells/well with 10,000 T cells of clone 14.5; thymidine incorporation was measured after 4 days of culture. (See ref. 183.)

The planar membrane technique has been applied to determine the minimum number of MHC–antigen complexes per antigen-presenting cell necessary to induce T cell activation (186). After pulsing the presenting cells with antigen, the cells were studied for antigen-presenting activity, and some of the cells were lysed to produce a purified fraction containing MHC charged with antigenic peptides. These MHC–peptide complexes were used to reconstitute planar membranes, and their potency was compared to a reference MHC preparation pulsed with a high peptide concentration *in vitro* and presumed to be fully loaded. In this way, the relative peptide occupancy of MHC binding sites corresponding to any level of antigen presentation could be determined. For B cells and macrophages, the threshold of antigen loading necessary for triggering T cells was 0.2% of I-E^d molecules occupied by peptide, corresponding to about 200 MHC–peptide complexes per presenting cell. For artificial presenting cells, such as L cells transfected with I-E^d, the threshold was 23 times greater, or 4.6% of MHC occupied by peptide. These data suggest an advantage in presentation by the natural presenting cells. In addition, the B cells required a 100-fold lower antigen concentration in the medium to generate the same level of MHC–peptide complexes, suggesting more efficient processing than macrophages, possibly due to excessive peptide degradation in macrophages. Similarly, when MHC–peptide binding was measured directly, using radiolabeled peptide to determine the minimum level of MHC–peptide complexes required for T cell triggering, B cells were capable of presenting antigen with as few as 200 to 300 MHC–peptide complexes per cell (187). A similar number of peptide–MHC class I molecule complexes was reported to be required on a cell for recognition by $CD8^+$ cytotoxic T cells (188). These results explain how newly generated peptide antigens can compete with all the rest of cellular antigens, since a low level of MHC occupancy is sufficient. In addition, this threshold of presentation may explain how multivalent protein antigens, such as viral particles, with 100 to 200 protein copies each, can be over 10^3-fold more immunogenic than the corresponding monomers (189).

Why is antigen processing necessary? Recent experiments suggest that antigen processing may uncover functional sites that are buried in the native protein structure. For example, a form of intact myoglobin that has been partially unfolded through chemical modification can behave like a myoglobin peptide and can be presented by APC even in the presence of enough pro-

tease inhibitor or chloroquine to completely block the presentation of native myoglobin (183). Denatured lysozyme could also be presented without processing to one T cell clone (142). This result suggests that the requirement for processing may simply be a steric requirement, that is, to uncover the two sites needed to form the trimolecular complex between antigen and MHC and between antigen and T cell receptor. Thus unfolding may be sufficient without proteolysis, and proteolysis may simply accomplish an unfolding analogous to Alexander's approach to the Gordian knot.

The importance of antigen unfolding for T cell recognition and the ability of unfolding to bypass the need for antigen processing apply not only to small proteins the size of myoglobin and lysozyme but also to a range of sizes from small peptides to extremely large proteins. At one extreme, Lee et al. (190) found that even fibrinogen, of M_r 340,000, does not need to be processed if the epitope recognized is on the carboxy terminal portion of the α chain, which is naturally unfolded in the native molecule. At the other extreme, even a small peptide of only 18 amino acid residues, apamin, requires processing unless the two disulfide bonds that hold it in the native conformation are cleaved artificially to allow unfolding (191). Therefore large size does not mandate processing, and small size does not necessarily obviate the need for processing, at least for class II presentation. The common feature throughout the size range seems to be the need for unfolding. This evidence, taken together with the earlier data on unfolding of myoglobin and lysozyme, strongly supports the conclusion that unfolding, rather than size reduction, is the primary goal of antigen processing and that either antigen presentation by MHC molecules or T cell receptor recognition frequently requires exposure of residues not normally exposed on the surface of the native protein. This conclusion is supported by recent studies of peptides eluted from class II MHC molecules (see Antigen Interaction with MHC Molecules, below).

Processing Antigen for T Cells Restricted to Class I MHC Molecules

In contrast to class II-restricted T cells, it was widely assumed that class I-restricted T cells, such as cytolytic T cells (CTLs) specific for virus-infected cells, responded mainly to unprocessed viral glycoproteins expressed on the surface of infected cells. For example, this appeared to be the case for influenza virus, where the hemagglutinin was detected on the cell surface and where CTLs specific for the hemagglutinin were demonstrated. However, converse examples began to appear at about the same time that antigen processing was demonstrated for class II-restricted T cells. For example, influenza nucleoprotein (NP) was found to be a major target antigen for influenza specific CTLs, even though NP remains in the nucleus of infected cells and none is detectable on the cell surface (192). This led to speculation that antigen presentation in association with class I molecules may also depend on some form of antigen processing.

Using the cloned NP gene to transfect L cells, Townsend et al. (148) showed that the NP protein could be manipulated in various ways and still function as a good target for CTLs, even though NP lacks a leader sequence to direct it to the cell surface. In addition, using a series of truncated constructs of the NP gene, they showed that CTLs lyse cells expressing NP fragment 1 to 386 but not those expressing NP fragment 1 to 327, so the antigenic site must lie between 327 and 386 (148). This was confirmed when they showed that target cells that take up synthetic NP peptide 366 to 379 were lysed by NP specific CTLs (174). This constitutes evidence that antigen presented in association with class I molecules requires processing into antigenic fragments. Also, the demonstration that synthetic peptides could sensitize targets for CTLs introduced a powerful tool for mapping and studying CTL epitopes.

In light of the results with NP, additional experiments were performed with hemagglutinin to determine whether surface expression of the intact glycoprotein was really necessary for class I-restricted CTL recognition. Removal of the leader sequence from the hemagglutinin gene inserted into vaccinia virus results in a protein antigen that is expressed within infected cells but lacks the signals for transport to the cell surface. Nevertheless, target cells expressing leader-negative hemagglutinin were lysed equally well as those with surface hemagglutinin (175). Similar conclusions were drawn from anchor-negative mutants (193). Thus, even for hemagglutinin, surface expression of antigen is not required for antigenicity, implying that it is the processed antigen that stimulates a T cell response.

The antigen-processing pathway leading to class I presentation is quite different from the endosomal pathway leading to class II presentation. Unlike endosomal processing, the nonendosomal pathway is insensitive to chloroquine, NH_4Cl, and leupeptin. In some cases, different forms of the same antigen can be processed exclusively by one pathway or the other. For example, in one study, UV-inactivated influenza virus was processed by the endosomal pathway, while live virus was not (194). Cells exposed to UV-inactivated virus were recognized only by class II-restricted CTLs, while cells exposed to live virus were recognized by class I-restricted CTLs. A model was proposed in which endogenous synthesis of viral proteins led to nonendosomal processing, and the resultant peptides were displayed on the cell surface with class I MHC molecules. This pathway would be inaccessible to exogenous proteins, accounting for their failure to

be presented with class I antigens (194). In effect, the processing pathway would determine the class restriction.

For this simple model to hold, each step in a processing pathway would have to be tightly linked, so that no crossing of pathways could occur. Given two sources of antigen, endogenous synthesis and uptake of exogenous proteins, two processing systems, nonendosomal and endosomal, and two classes of MHC molecules, theoretically up to eight combinations might be possible. The simple model allows for just two combinations out of the eight: exogenous antigen being degraded in endosomes and the peptides binding to class II, or endogenously synthesized antigen being degraded nonendosomally and the resultant peptides binding to class I molecules. Over time, at least four additional combinations have appeared, which may be thought of as alternative paths leading into or out of the two processing systems.

First, exogenous antigens such as those from UV-inactivated influenza virus can enter the nonendosomal pathway for presentation with class I molecules when the viral neuraminidase is inhibited (195), perhaps due to increased antigen binding and entry into cells. This effect is accentuated at acid pH (196), under conditions which activate the membrane fusion activity of influenza hemagglutinin (197) and allow virus to penetrate directly across the plasma membrane. Thus direct entry of virus into the cytoplasm enables exogenous antigen to enter the nonendosomal processing pathway leading to MHC class I association. Similarly, experiments with HBsAg specific human T cell clones (198) have shown that exogenous HBsAg particles can gain access to nonendosomal processing, which may contribute to the potency of HBsAg vaccine. Another example showing that entry into the cytoplasm is sufficient to direct antigen into the nonendosomal pathway came from studies in which ovalbumin was taken up by cells under hyperosmotic conditions, followed by reducing the osmolality and lysing the pinocytotic vesicles into the cytoplasm. These cells presented ovalbumin fragments with class I molecules to $CD8^+$ CTL (199). As shown in these three cases, exogenous antigens may enter the nonendosomal pathway without being synthesized endogenously, depending on cytoplasmic entry, rather than the site of synthesis.

In a second new combination, endogenously synthesized antigen can enter endosomal processing for presentation with class II. Endogenously synthesized HBsAg can be processed by the endosomal pathway and recognized by $CD4^+$ T cell clones in association with MHC class II (198). Similarly, Weiss and Bogen have shown that endogenously synthesized λ light chains can be recognized by class II-restricted T cells (200,201), and Eisenlohr and Hackett made a similar finding for recognition of influenza neuraminidase by a class II-restricted T cell hybridoma (202). In some of these cases, endosomal processing might be explained as the reentry into endosomes of antigens expressed on the cell surface. However, Polydefkis et al. (203) reported that endogenous gp160 of HIV-1 is recognized in its nonsecreted form by class II-restricted T cells, but not in its processed and secreted form. Similarly, Jaraquemada et al. (204) reported the class II associated recognition of endogenously synthesized nonsecreted influenza virus M1 matrix protein after endosomal processing. In some cases, the endogenous antigen was expressed by cells infected with a recombinant vaccinia virus containing the appropriate gene (205), and the possibility of exogenous antigen contaminating the viral preparation was excluded by using purified virus and by showing that UV inactivation of the virus blocked antigen presentation by the infected cells.

A third and somewhat more controversial new combination is that of exogenous proteins processed by the nonendosomal processing pathway and then presented by MHC class II. For example, Nuchtern et al. (196) have studied class II-restricted CTL lines that recognize antigenic fragments of influenza matrix protein after nonendosomal processing, as shown by sensitivity to brefeldin A (an inhibitor of Golgi traffic) and resistance to chloroquine. Similarly, Jacobson et al. (206) reported class II-restricted CTL against measles virus proteins that were chloroquine insensitive, and Y. Jin and I. J. Berkower (*unpublished results*) have found several class II-restricted T cell clones that recognize exogenous HBsAg after nonendosomal processing (chloroquine and leupeptin insensitive). It should be noted that the only difference between this third combination and the classical pathway for exogenous antigen presented by class II MHC molecules is the resistance in each case of the processing to chloroquine inhibition. A possible fourth new combination, in which endogenously expressed HBsAg was presented with class II after nonendosomal processing, was also observed for the same HBsAg specific T cell clones. Given these many exceptions, a new paradigm may be needed to explain antigen entry and exit from the two processing systems and the subsequent presentation of peptides by classes I and II. Cytoplasmic entry, rather than endogenous protein synthesis, is the common denominator for nonendosomal processing. Endocytosis of exogenous proteins favors endosomal processing, although some antigens may escape endosomes and proceed to nonendosomal processing. Special signals on viral proteins, such as membrane fusion peptides used by the virus to escape from endosomes, may affect localization and processing. After processing, various cellular proteins play an important role in directing the peptide products toward class I or II, based on entry into cellular compartments where MHC binding sites are accessible to peptides.

Important information on how processing for presentation by class I MHC molecules could occur has been obtained by studies of mutants with low level expression of MHC class I, including the mutant murine cell line RMA-S (207) and human B cell lines 721.174 and 721.134 (208). The class I molecules are synthesized, but they remain trapped in the endoplasmic reticulum, without proper glycosylation and not associated with β2-microglobulin, or they reach the cell surface in an unstable complex with β2-microglobulin, only to be degraded rapidly. As shown by Townsend et al. (209), these cells fail to process and present endogenously expressed viral antigens by class I for CTL recognition. However, both of these functions, MHC expression and CTL recognition, can be rescued by incubation with class I binding peptides.

Genetic analysis of these mutants revealed homozygous large or small deletions of part of the MHC class II region near the D_R locus, demonstrating that a function coded in the class II region controls cell surface expression and function of class I proteins. Molecular cloning of DNA from this region revealed at least 12 new genes, of which two, called TAP-1 and TAP-2, for "transporter associated with antigen processing," showed a typical sequence for transporter proteins (210–212), while two others, LMP-2 and LMP-7, appeared to be part of the processing machinery. The TAP protein sequences are characterized by membrane spanning and ATP binding domains and sequence homology to other peptide transport proteins such as Opp D in salmonella and the multidrug resistant protein and the cystic fibrosis transporter in humans. Presumably, their function is to transport processed peptides from the nonendosomal processing pathway to the endoplasmic reticulum. Once in this compartment, peptides bind to newly formed MHC class I molecules and stabilize a trimolecular complex with β2-microglobulin. This complex is then transported to the cell surface, where antigen presentation occurs. Without the peptide transporters, empty dimers of MHC class I with β2-microglobulin form, but these are unstable. Excess free peptide would rescue MHC class I by stabilizing the few short-lived empty complexes that reach the surface, as shown by Townsend et al. (209) and Schumacher et al. (213). Even uninfected cells need a supply of MHC binding self-peptides to produce stable MHC class I complexes and maintain normal levels of MHC on their surface. The function of the TAP proteins was demonstrated by reconstituting mutant cells with the cloned genes for each transport protein. Transfection with TAP-2 restores the antigen-presenting ability of RMA-S (214), while transfection with TAP-1 restores it for 721.134 (215). Mutant 721.174, with both TAP-1 and TAP-2 deleted, cannot be returned to normal antigen presentation with either TAP gene. The TAP-1 and TAP-2 proteins appear to be physically associated in a heterodimer, since antipeptide antibodies specific for either protein will coprecipitate both (215,216).

Assuming that these proteins form the peptide transporter between nonendosomal processing and the endoplasmic reticulum, they would play an important role in MHC function. If they are highly efficient, transporting most of the peptides produced, nonendosomal processing will be tightly coupled to class I. If less efficient, some of the nonendosomal peptides may be available for class II binding. It is not yet clear whether such nonendosomal peptides must first travel to the cell surface to be recycled into endosomes, or whether there may be transport mechanisms not yet identified for transport directly into endosomes, where class II binding can occur. A second effect of transporter proteins may be to limit which peptides can be transported into the endoplasmic reticulum for presentation with class I, and genetic polymorphism of the TAP proteins suggests that different individuals may transport different peptides (217). This would include self-peptides as well as foreign, since MHC class I expression on uninfected cells also depends on peptide transport (209), and thus could affect autoimmunity.

Additional studies of the deletion mutant 721.174 revealed two new genes, known as LMP-2 and LMP-7 for "low molecular weight protein," located close to the TAP-1 and TAP-2 genes. These proteins assemble with about 14 others to form a high molecular weight complex of about 580 kD. Due to MHC-linked polymorphism of these two LMP proteins, polyclonal anti-MHC sera bind LMP-2 and LMP-7 and precipitate the entire complex of MHC-linked and nonlinked proteins (218). A very similar complex was described by Tanaka et al. (219,220) and Arrigo and co-workers (221), who found a 650-kD protease of the cytosol, which had at least three catalytic sites and a mixed protease activity. This activity was increased against aberrant proteins, including those containing amino acid analogs, and the products were peptides, including nonamers. The protease was identified with a particle, termed the "proteasome," that occurs in close proximity with the rough endoplasmic reticulum (ER). The LMP proteins and proteasome are closely related, since antiserum against the LMP complex and antiserum against the proteasome cross-react with the same complex (222), although the extent of precipitation varied, depending on which antiserum was used. All LMP complexes contain proteasome proteins, but only 5% to 10% of proteasomes contain LMP-2 and LMP-7. Currently, the proteasome is the leading candidate for the nonendosomal processing machinery, mainly because of the genetic linkage of LMP-2 and LMP-7 to the TAP genes, but the function of LMP-2 and LMP-7 is unclear. If proteasomes containing LMP-2 and LMP-7 are coupled to the peptide transporter, as would be required for tight linkage to MHC class I, then 90% of

proteasomes lacking these proteins would be open to other possibilities for coupling.

In an infected cell, as soon as viral proteins are made, peptide fragments generated by the proteasome become available to the TAP-1 and TAP-2 transporter proteins. These transport the peptide fragments into the endoplasmic reticulum for association with newly formed MHC class I molecules, which would carry them to the cell surface for antigen presentation, all within 30 min. Thus MHC-linked genes coding for proteolysis, peptide transport, and presentation at the cell surface have been identified. In effect, the MHC now appears to encode a complex system of multiple elements devoted to the rapid display of foreign protein determinants on the surface of an infected cell. By continuously sampling the output of the protein synthesizing machinery, this system permits rapid identification and destruction of infected cells by CTL before infectious virus can be released.

In this light, it is apparent that despite the evidence for cross-talk between the class I and class II antigen processing pathways, there is good teleological reason for the pathways to be kept separate. Class I MHC molecules present antigen to $CD8^+$ T cells, which are usually cytotoxic, and provide immune surveillance to destroy cells producing inappropriate proteins endogenously, such as viral proteins in a virus-infected cell or mutant proteins or other tumor antigens in a cancer cell. In contrast, class II molecules present antigen to $CD4^+$ T cells, which generally activate the cells with which they interact, such as a B cell to make antibody, or a macrophage to activate its cytolytic machinery. It would not benefit the organism if a B cell, which had been waiting all its life to encounter the antigen for which it expressed specific immunoglobulin, finally encountered that antigen only to present it to a CTL and be killed, rather than to a helper T cell to be activated. Similarly, it would be counterproductive for a virus-infected cell to present viral antigens to a helper T cell and thereby become activated to produce more virus, instead of being killed by a CTL. It is this dichotomy that may explain the evolution of two distinct classes of MHC molecules and correspondingly two distinct pathways of antigen processing to provide for both needs for sampling foreign antigens but keeping them separate.

Influence of Antigen Processing on the Expressed T Cell Repertoire

Several lines of evidence indicate that antigen processing plays a critical role in determining which potential antigenic sites are recognized and therefore what part of the potential T cell repertoire is expressed upon immunization with a protein antigen. Since the T cell does not see the native antigen but only the products of antigen processing, it is not unreasonable that the nature of these products would at least partly determine which potential epitopes could be recognized by T cells.

One line of evidence that processing plays a major role in T cell repertoire expression came from comparisons that were made of the immunogenicity of peptide versus native molecule in the cases of myoglobin (223) or lysozyme (224). In the case of myoglobin, a site of equine myoglobin (residues 102 to 118) that did not elicit a response when H-2^k mice were immunized with native myoglobin nevertheless was found to be immunogenic when the mice were immunized with the peptide (223). Thus the low responsiveness to this site in mice immunized with the native myoglobin was not due to either of the classical mechanisms of *Ir* gene defects—namely, a hole in the T cell repertoire or a failure of the site to interact with MHC molecules of that strain. However, the peptide-immune T cells responded only poorly to native equine myoglobin *in vitro*. Thus the peptide and the native molecule did not cross-react well in either direction. The problem was not simply a failure to process the native molecule to produce this epitope, because (H-2^k × H-2^s)F_1-presenting cells could present this epitope to H-2^s T cells when given native myoglobin but could not present it to H-2^k T cells. Also, because the same results applied to individual T cell clones, which should not be contaminated with suppressor cells, the failure to respond to the native molecule was apparently not due to suppressor cells induced by the native molecule. Similar observations were made for the response to the peptide 74 to 96 of hen lysozyme in B10.A mice (224). The peptide, not the native molecule, induced T cells specific for this site, and these T cells did not cross-react with the native molecule. With the above alternative mechanisms excluded, we are left with the conclusion that an appropriate peptide was produced but it differed from the synthetic peptide in such a way that a hindering site outside the minimal antigenic site interfered with presentation by presenting cells of certain MHC types.

Further evidence consistent with this mechanism came from the work of Shastri et al. (225), who found that different epitopes within the 74 to 96 region of lysozyme were immunodominant in H-2^b mice when different forms of the immunogen were used. With native hen lysozyme as immunogen, the T cells specific for this region all responded to 74 to 90 but not to 81 to 96, whereas with the cyanogen bromide fragment 13 to 105 as immunogen, the T cells that were elicited responded to 81 to 96, not 74 to 90. If the tryptic fragment 74 to 96 was used for immunization, both sets of T cells were elicited.

Another line of evidence came from fine specificity studies of individual T cell clones. Shastri et al. (226) observed that H-2^b T cell clones specific for hen lyso-

zymes were about 100-fold more sensitive to ring-necked pheasant lysozyme than to hen lysozyme. Nevertheless, they were equally sensitive to the cyanogen bromide cleavage fragments containing the antigenic sites from both lysozymes. Thus regions outside the minimal antigenic site removable by cyanogen bromide cleavage presumably interfered with presentation or recognition of the corresponding site in hen lysozyme or with processing of the native molecule to produce these sites. Similarly, it was observed that a T cell clone specific for sperm whale myoglobin, not equine myoglobin, responded equally well to the minimal epitope synthetic peptides from the two species (223). Here too residues outside the actual site must be distinguishing equine from sperm whale myoglobin. It is possible that equine myoglobin is processed differently from sperm whale, so that the fragment containing the site is poorly produced. However, experiments using F_1-presenting cells that can clearly produce this epitope for presentation to other T cells proved that the problem was not a failure to produce the appropriate fragment from hen lysozyme (224) or equine myoglobin (223). Thus these cases provide evidence that a structure outside the minimal site can hinder presentation in association with a particular MHC molecule.

Even a small peptide that does not need processing may nevertheless be processed, and that processing may affect its interaction with MHC molecules. Fox et al. (227) found that substitution of a tyrosine for isoleucine at position 95 of cytochrome *c* peptide 93 to 103 enhanced presentation with E^b_β but diminished presentation with E^k_β when live APCs were used but not when the APCs were fixed and could not process antigen. Therefore the tyrosine residue was not directly interacting with the different MHC molecule but was affecting the way the peptide was processed, which in turn affected MHC interaction.

Besides the mechanisms suggested above, Gammon et al. (224) and Sercarz et al. (228) have proposed the possibility of competition between different MHC-binding structures ("agretopes") within the same processed fragment. If a partially unfolded fragment first binds to MHC by one such site already exposed, further processing may stop, and other potential binding sites for MHC may never become accessible for binding. Such competition could also occur between different MHC molecules on the same presenting cell (224). For instance, BALB/c mice, expressing both A^d and E^d, produce a response to hen lysozyme specific for 108 to 120, not for 13 to 35 (224), and this response is restricted to E^d. However, B10.GD mice that express only A^d respond well to 13 to 35 when immunized with lysozyme. BALB/c mice clearly express an A^d molecule, so the failure to present this 13 to 35 epitope may be due to competition from E^d, which may preempt by binding the 108 to 120 site with higher affinity and preventing the 13 to 35 site from binding to A^d. Competition between different peptides binding to the same MHC molecule could also occur.

All these results, taken together, indicate that antigen processing not only facilitates interaction of the antigenic site with the MHC molecule and/or the T cell receptor but also influences the specificity of these interactions and in turn the specificity of the elicited T cell repertoire. These considerations will be important in the development of synthetic vaccines, as it is essential to use peptides capable of eliciting T cells that can be recalled by the native molecule on the pathogen.

Intrinsic Structural Features Contributing to Immunogenicity for T Cells

As just discussed, T cells recognize protein antigens only on the surface of another cell, and only after processing, which involves either cleavage into small fragments or at least unfolding. This fact leads to the ironic situation that T cell recognition of antigen, which is more complex than antibody recognition due to the ternary complex needed between T cell receptor, antigen, and MHC molecule, may actually be focused on simpler structures than those seen by most antibodies specific for native protein antigens. In contrast to the assembled topographic antigenic sites seen by many antibodies (40,41), T cells specific for processed antigens are limited to seeing short segments of continuous sequence (135,229). Therefore the tertiary structure of the protein plays little if any role in the structure of the epitope recognized by T cells, although it may play a more distant role in determining the way the protein is processed and therefore indirectly influence which epitopes are available for T cell recognition, as discussed previously. However, the structure of the T cell antigenic site itself must be limited to primary (sequence) and secondary structure, the latter depending only on local rather than long-range interactions. This limitation greatly simplifies the problem of identifying structural properties important to T cell recognition, because one can deal with sequence information, which can now be obtained from DNA without having a purified protein, and with the secondary structure implicit therein without having to obtain an x-ray crystallographic three-dimensional structure of the native protein, a much more difficult task.

These features have greatly facilitated the structural analysis of antigenic sites recognized by T cells by allowing the use of short synthetic peptides and recombinant fragments that do not retain the native structure of the protein. The comparative ease of producing overlapping and truncated peptides and sequence variants, combined with the limitation of the search for salient features to primary and secondary structure, has led to a major

surge in efforts over the past few years to identify properties that will be useful in predicting the location of antigenic sites.

The search has especially focused on sites that may be immunodominant when an individual is exposed to or immunized with the native protein. In contrast to antibodies that bind all over the surface of a native protein (40) (see section entitled Protein and Polypeptide Antigenic Determinants), it has been observed that T cells elicited by immunization with the native protein tend to be focused on one or a few immunodominant sites (230,231). This is true whether one deals with model mammalian or avian proteins such as cytochrome *c* (137), myoglobin (136,138), lysozyme (140,161,229, 232), insulin (144,165), and ovalbumin (141), or with bacterial, viral, and parasitic proteins from pathogens, such as influenza hemagglutinins (163) or nucleoprotein (174), staphylococcal nuclease (233), or malarial circumsporozoite protein (108,234). Because the latter category of proteins shares no obvious homology to mammalian proteins, the immunodominance of a few sites cannot be attributed simply to tolerance for the rest of the protein because of self-tolerance to homologous host proteins. Moreover, immunodominance is not simply the preemption of the response by a single clone of predominant T cells, because it has been observed that immunodominant sites tend to be the focus for a polyclonal response of a number of distinct T cell clones recognizing overlapping subsites within the antigenic site or having different sensitivities to substitutions of amino acids within the site (140,141,145,146,160,161).

Immunodominant antigenic sites appear to be qualitatively different from other sites. For example, in the case of myoglobin, when the number of clones responding to different epitopes after immunization with native protein was quantitated by limiting dilution, it was observed that the bulk of the response to the whole protein in association with the high-responder class II MHC molecules was focused on a single site within residues 102 to 118 (235) (Fig. 9). There were T cells specific for other epitopes, but their numbers never reached the same level as those specific for the immunodominant site. Moreover, when T cells (from the same immunization of F_1 hybrid mice) specific for the protein associated with the low-responder class II MHC molecules were compared to those discussed above, the response to nonimmunodominant sites was quantitatively similar to that for the same sites restricted to the high-responder MHC. The major difference was the absence of a response specific for the immunodominant site in association with the low-responder MHC (Fig. 9). Similar results were found for two different high-responder and two different low-responder MHC haplotypes (235). Thus *Ir*-gene-controlled high or low responsiveness could be accounted for by the presence or absence of a response to

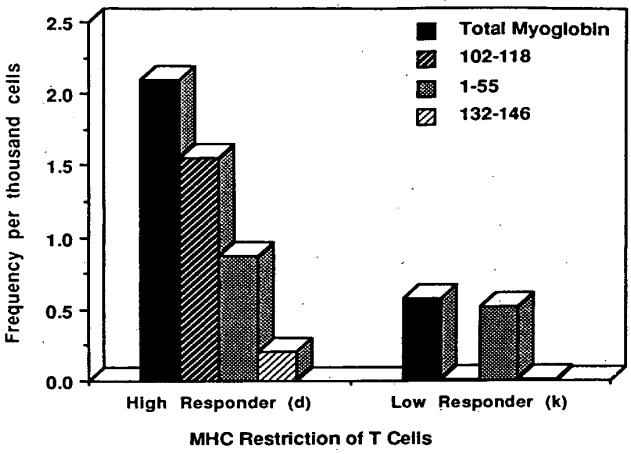


FIG. 9. Frequency of high- and low-responder MHC-restricted T cells in F_1 hybrid. High responsiveness may be accounted for by the response to a single immunodominant epitope. Lymph node T cells from [low-responder (H-2^k) × high-responder (H-2^d)]F_1 hybrid mice immunized with whole myoglobin were plated at different limiting dilutions in microtiter wells with either high-responder or low-responder presenting cells and myoglobin as antigen. The cells growing in each well were tested for responsiveness to whole myoglobin and to various peptide epitopes of myoglobin. The frequency of T cells of each specificity and MHC restriction was calculated from Poisson statistics and is plotted on the ordinate. Most of the difference in T cell frequency between high- and low-responder restriction types (*solid bars*) can be accounted for by the presence of T cells responding to the immunodominant site at residues 102 to 118, accounting for more than two-thirds of the high-responder myoglobin specific T cells, in contrast to the absence of such T cells restricted to the low-responder MHC type. (Based on the data in ref. 235.)

this immunodominant site, even though all haplotypes responded to some antigenic sites. Why didn't the response to the other sites compensate for the lack of response to the immunodominant site in the low responders? It appears that there is something special about the immunodominant site. The greater frequency of T cells specific for the immunodominant site may in part be attributed to the large number of ways this site can be recognized by different T cell clones, as mentioned above, but this only pushes the problem back one level. Why is an immunodominant site the focus for so many different T cell clones? Since the answer cannot depend on any particular T cell, it must depend on other factors. These can be divided into those intrinsic to the antigenic site and those extrinsic to the site (230,231).

A major factor extrinsic to the site is the MHC of the responding individual (138,165,236). Identification of structural parts of the peptide responsible for binding to MHC and attempts to generalize from these identifications to predict which peptide will bind to which MHC

molecule are the subject of the next section on antigen interaction with MHC.

However, there are other factors extrinsic to the antigenic site itself but not extrinsic to the protein molecule as a whole. It has been observed that many peptides may be immunogenic themselves, but the T cell response they elicit is specific only for the peptide and does not cross-react with the native protein (223,224). Conversely, not all peptides that are immunogenic themselves will correspond to immunogenic sites when the native protein is used as immunogen (223,224). The reasons for these differences may involve the way the native protein is processed to produce fragments distinct from but including or overlapping the synthetic peptides used in experiments, and also the competition among sites within the protein for binding to the same MHC molecules, as discussed in the preceding section.

Identification of factors intrinsic to the antigenic site itself has been facilitated, as noted above, by the ability to focus on just the primary and secondary levels of structure. The first structural feature of an immunodominant T cell site was identified in a series of studies of a single such site at the carboxyl terminus of pigeon cytochrome *c* (145,177,180). It was observed that for a series of peptides from this region of different lengths and variant sequences, the potency in stimulating T cell clones and hybridomas correlated with their predicted (177) or measured (145,180) ability to fold as an α helix. Subsequent statistical analysis by Spouge et al. (179) of a database of 23 immunodominant sites demonstrated that this property was significantly associated with T cell immunodominance.

Several other studies of individual determinants using panels of variant peptides with single amino acid substitutions at each position in the sequence also suggested that the peptide bound to the class II MHC molecule as an α helix (237–240), although others did not (241). This seemed consistent with the initial model of class II MHC structure (242) based on the crystal structure of class I MHC molecules (243,244), in which it appeared that a helical peptide would make a perfect fit between the antiparallel α helices that bounded the peptide binding groove. However, subsequent crystal structures of peptides bound to class I MHC molecules indicate that these are generally bound as extended structures (245–247), and a study of peptides eluted from class II MHC molecules was interpreted as implying the same for peptides bound to class II molecules (248). Therefore it may be that helicity contributes at some stage other than the actual binding of peptide to MHC molecule.

Helical amphipathicity (173,178,179,249,250) is another secondary structural feature of immunodominant T cell sites that, although associated with helicity, is statistically significant independent of the tendency to form a helix (179). *Amphipathicity* is the property of having hydrophobic and hydrophilic regions separated in space (Fig. 10). It was observed that both of the immunodominant T cell epitopes for myoglobin corresponded to amphipathic helices (143,146). To see whether this observation was peculiar to myoglobin, which has a high helical content, or whether it was true of immunodominant T cell antigenic sites in other proteins as well, DeLisi and Berzofsky (178) developed an algorithm to search for segments of protein sequence that could fold as amphipathic helices. The approach was based on the idea that, as hydrophilicity is the negative of hydrophobicity, the hydrophobicity of the amino acids in the sequence must oscillate as one goes around an amphipathic helix. For the hydrophobic residues to line up on one side and the hydrophilic residues on the other, the periodicity of this oscillation must be approximately the same as the structural periodicity of the helix (Fig. 11). For an α helix, this is 100° per turn (360°/3.6 residues per turn) and for a 3_{10} helix, this is 120° per turn (360°/3 residues per turn).

A microcomputer program implementing this analysis was published (250). Subsequently, Margalit et al. (173) optimized the original approach (178) by comparing a number of related algorithms and found that, for short segments, a least-squares best fit to a sinusoidal function (252) was more effective than the Fourier trans-

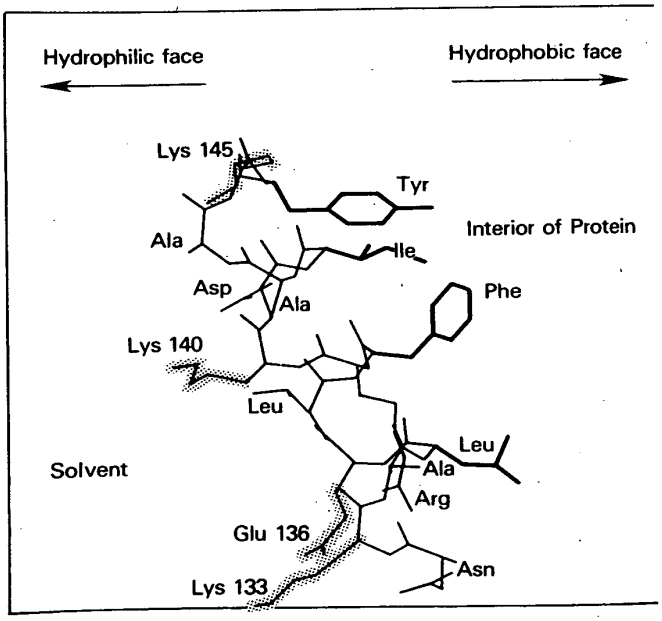


FIG. 10. Peptide 132 to 146 is shown in the α helical structure that is found in native myoglobin. In this conformation, the hydrophobic side chains line up on one side of the helix, and the hydrophilic side chains line up on the other side, forming at least two types of potentially functional domains for possible interactions with cell surface receptors. The three critical residues identified in Berkower et al. (143), Glu 136, Lys 140, and Lys 145, are one such domain and are brought together by successive turns of the α helix. (From ref. 143, with permission.)

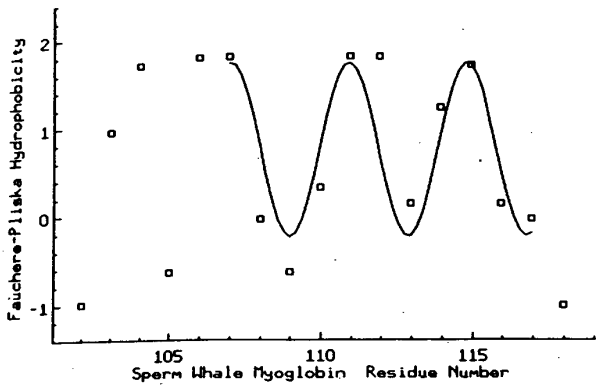


FIG. 11. Plot of hydrophobicity of each amino acid in sperm whale myoglobin 102 to 118, according to the scale of Fauchère and Pliska (251), as a function of amino acid sequence, showing least-squares fit of a sinusoidal function to the sequence of hydrophobicities from 107 to 117. (From ref. 138, with permission.)

form. This algorithm correctly identified 18 of the 23 immunodominant helper T cell antigenic sites seen in association with class II MHC molecules from the 12 proteins in an expanded database ($p < 0.001$) (173) (Table 6). Indeed, when newly discovered helper T cell determinants were included to expand the database to twice and then four times its original size, the correlation remained highly significant, and the fraction of sites predicted remained relatively stable (34/48 sites = 71%, $p < 0.003$; 61/92 sites = 66%, $p < 0.001$) (253,254). However, it is clear that a sizable fraction of determinants do not have this property.

At the time of the initial studies, very few peptides presented by class I MHC molecules to $CD8^+$ CTL had been identified. However, subsequent analysis of 51 peptides presented by class I molecules indicated that 33 (65%) could fold as amphipathic helices ($p = 0.05$) (J. L. Cornette, H. Margalit, C. DeLisi, and J. A. Berzofsky, *unpublished results*). Moreover, 7 of 11 natural endogenous peptides eluted from HLA-B27 by Jardetzky et al. (255) had this property, compared to 2/11 or 3/11 for control sets of peptides ($p = 0.02$) (254). Thus it appears that a similar fraction of sites presented by class I MHC molecules can also fold as amphipathic helices. Another approach, called the "strip-of-the-helix" algorithm, that searches for helices with a hydrophobic strip down one face, also found a correlation between amphipathic helices and determinants presented by both class II and class I MHC molecules (249,256).

At least four explanations for these observations have been suggested. First, it is possible that an amphipathic helix binds particularly well in the binding site of the MHC molecule. However, as pointed out above, the crystallographic data for the three cases of peptides bound to class I molecules studied so far all suggest that the peptide is bound in an extended structure (245–247). Although class II molecules may have more freedom to bind peptides as helices, pending the crystal structures, these data suggest that in most cases peptides are forced to assume extended structures when bound to MHC molecules.

A second independent, but not mutually exclusive, explanation is the ability of amphipathic helices to intercalate into membranes (257–259). As the affinity of the MHC molecules for peptide is rather low [on the order of $10^5\ M^{-1}$ (260,261)], peptides that remain associated with the plasma membrane or the endosomal membrane after processing may be present in higher local concentration to push the equilibrium for binding to MHC and therefore may have a better chance to bind than those that do not associate with the membrane (138,262). A corollary of this hypothesis is that the membrane may function as a short-term memory of the peptides from all the protein antigens that the APC has encountered over some period of time. The MHC molecules may constantly be sampling from that reservoir, and only when the appropriate T cell is present would the interaction be productive (138,262,263). This idea is also consistent with the observation of Falo et al. (263,264) that lipase can strip antigen from the surface of the APC and inhibit presentation of that antigen without interfering with the general function of the cell, as measured by its ability to subsequently present other antigens.

A third explanation also related to the interaction of amphipathic structures with membranes was recently suggested by A. Singer (A. Singer, M. Gottesman, W. Biddison, J. A. Berzofsky, and W. Shores, *unpublished results*), based on the observation that all known transport proteins studied of the same class that transports peptides from the cytoplasm to the endoplasmic reticulum, such as the multidrug resistant proteins, transport specifically amphipathic structures (265,266). Therefore, in the case of endogenous peptides that require such transport, ones that are amphipathic should be favored at the transport stage. Indeed, this hypothesis also may explain the clustering of determinants (267) observed, because determinants that are not themselves amphipathic should be associated on processed fragments that are amphipathic. Although this hypothesis is very compelling for class I, it does not clearly explain the correlation for class II unless a similar transport step is required.

A fourth explanation for the importance of amphipathic helicity is that such helices may be less susceptible to proteolytic degradation and therefore may escape destruction during processing (as suggested by J. Rothbard and O. Werdelin, *personal communications*). Whatever the explanation, the correlation of immunodominance with amphipathic helical structure may contribute to models of peptide binding to MHC and may have practical use in predicting the location of potential immunodominant T cell antigenic sites for vaccine design (138).

It has already been used successfully to predict antigenic sites in the malaria circumsporozoite protein (108,234), a malaria merozoite protein (268), the AIDS viral envelope protein (269), and the acetylcholine receptor (270).

Other intrinsic properties that might be correlated with significantly immunodominant antigenic sites seen in association with class II MHC molecules were examined by Spouge et al. (179). Using a Monte Carlo statistical analysis of parameters designed to model folding free energy in various environments and using matching to test for independence of variables, they found that helical amphipathicity and α helicity were both statistically significant properties independent of each other; that is, neither one was significant simply because it was correlated with the other. Segmental amphipathicity, the property of having a stretch of hydrophilic amino acid sequence adjacent to a stretch of hydrophobic sequence, was not at all significant, despite the presence of a few examples of antigenic sites that had this property. Neither the tendency to form a β strand nor β-strand amphipathicity was significant. Another property was noted that surprisingly remained significant independent of any of the other properties (as assessed by matching). This was the presence of lysine residues near the carboxyl terminus. Although a positive charge near the carboxyl terminus can help to stabilize an α helix by countering the helical dipole (271–273), its significance independent from helicity itself indicated that the explanation for this lysine correlation was not simply in stabilizing helices, although that could be one factor. The primary mechanism of this lysine effect remains unknown but may become apparent when the structure of the binding site of class II MHC molecules is known. In this regard, it is of interest that cationization of proteins by converting carboxyl groups to aminoethyl-amide groups makes them more immunogenic *in vivo* and increases the potency of their T cell stimulation *in vitro* by several orders of magnitude (274,275). It may be that a positive charge in the right location enhances binding to the APC or the T cell receptor.

Rothbard and Taylor (276) took another approach, namely, searching for common patterns in the sequence of T cell antigenic sites. They observed that a very high percentage of sites seen with class I or class II MHC molecules contained either a four-residue pattern (charged residue or glycine, hydrophobic, hydrophobic, polar) or a five-residue pattern (similar, but with three hydrophobic residues in a row). For these patterns to fit the maximum number of sites, threonine and tyrosine had to be taken as either hydrophobic or polar in different cases. It is hard to determine rigorously the statistical significance of this pattern, but it was striking that, in several cases where the minimum size of the epitope was determined experimentally, the four- or five-residue pattern was contained intact. No physical interpretation of these patterns has been suggested, but it is also interesting that this sequence pattern looks like one turn of an amphipathic α helix so that they may have the same structural basis. This pattern approach has also been used successfully to locate sites in untested proteins.

The ability to synthesize and study large numbers of peptides (45,46,152) will facilitate the testing of these properties of antigenic sites and the detection of others. Already such large-scale peptide synthetic approaches are being used to map precisely T cell antigenic sites and determine the functional role of each residue, as is discussed in the next section.

Antigen Interaction with MHC Molecules

The response of T cells to antigens on APCs or target cells provided a number of hints that antigen interacts directly or indirectly with MHC molecules of the APCs. First, inheritable genes coding for immune responsiveness to a specific antigen are tightly linked to the inheritance of genes for MHC-encoded cell surface molecules (99,129). Second, it became apparent that T cell recognition of antigen is the step at which MHC restriction occurs (99,137,165,236). For example, *in vitro* T cell responses to small protein and polypeptide antigens were found to parallel *in vivo* responses controlled by *Ir* genes, and T cells were exquisitely sensitive to differences in MHC antigens of the APC in all their antigen recognition functions. This discovery was an important advance, because it became possible to separate the MHC of the T cell from that of the APC. The T cell response to antigenic determinants on each chain of insulin depended on the MHC antigens of the APC. This was particularly apparent when $(A \times B)F_1$ T cells responded to antigen presented by APCs of either the A or B parental MHC type (165,277). Neither parental APC stimulated an allogeneic response, and the response to antigen was as much limited by the MHC of the APC as by the T cell specificity. This ability of the APC to limit what could be presented to the T cells was termed "determinant selection" (165,277). It became obvious that even in a single $(A \times B)F_1$ animal, distinct sets of antigen specific T cells exist that respond to each antigenic determinant only in association with MHC type A or type B (278).

Experiments on the fine specificity of antigen specific T cell clones suggested that the MHC of the APC could influence the T cell response in more subtle ways than just allowing or inhibiting it. Determinant selection implied that a given processed peptide should contain both a site for MHC interaction and a distinct functional site for T cell receptor binding. The logical consequence of this is that different peptides processed from the same protein could have different MHC restrictions, due to different MHC association sites on each, consistent with the independent *Ir* gene control of the response to each antigenic determinant on the same protein (139). For example, T cell clones specific for myoglobin responded

to multiple antigenic determinants on different peptide fragments of myoglobin (158): those specific for one of the epitopes were always restricted to I-A, while those specific for the other were always restricted to I-E (Table 7). The simplest interpretation was that each antigenic peptide contained an Ia association site for interacting with I-A or I-E. At the level of *Ir* genes, mouse strains lacking a functional I-E molecule could respond to one of the sites only, and those with neither I-A nor I-E molecules capable of binding to any myoglobin peptide would be low responders to myoglobin.

Evidence for a discrete MHC association site on peptide antigens came from studies with pigeon cytochrome *c*. The murine T cell response to pigeon cytochrome *c* and its carboxy terminal peptide (81–104) depends on the I-E molecules of the APCs (137). However, distinct structural sites on the synthetic peptide antigen appear to constitute two functional sites: an epitope site for binding to the T cell receptor and an "agretope" (for "*a*ntigen-*r*estriction-tope") site for interacting with the MHC molecule of the APC (137,149–151). Amino acid substitutions for Lys at position 99 on the peptide destroyed the ability to stimulate T cell clones specific for the peptide, while the difference between Ala and a deletion at position 103 determined T cell stimulation in association with some MHC antigens but not others, independent of the T cell fine specificity. In addition, immunizing with the peptides substituted at position 99 elicited new T cell clones that responded to the substituted peptide but not the original and showed the same pattern of genetic restriction, correlated with the residue at position 103, as the clones specific for the original peptide. These results implied that the substitutions at position 99 had not affected the MHC association site but independently altered the antigen subsite that interacts directly with the T cell receptor. In contrast, position 103 was a likely subsite for MHC interaction, without altering the T cell receptor binding site. This interpretation is summarized in a model in Fig. 12.

TABLE 7. *Myoglobin specific T cell clones*[a]

MHC class II restriction	Epitope specificity	
	Glu 109	Lys 140
I-A^d	9.8	—
	9.23	
	9.24	
	9.27	
	13.15	
F_1D2	1.2	
I-E^d		9.15
		9.21
		13.9
		13.11
		14.1
		14.2
		14.4
		14.5
		14.6

From ref. 158, with permission.

[a] Each clone listed responds to one or the other epitope in association with I-A or I-E. Clonal specificity for epitopes of myoglobin correlates with the requirement for MHC class II of the antigen-presenting cell.

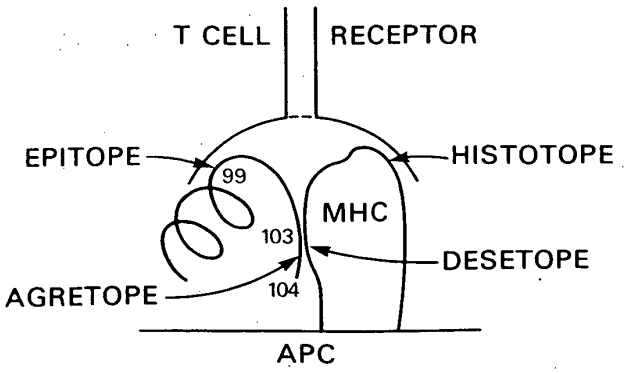


FIG. 12. A trimolecular complex type of model showing the interaction of antigen, the MHC-encoded product on the antigen-presenting cell, and the receptor on the T cell. The antigen shown is pigeon cytochrome *c* fragment 81 to 104. Residues 99 and 103 or 104 are highlighted because of evidence suggesting that for this antigen they might be part of the epitope and agretope, respectively. (From ref. 149, with permission.)

It remained to be shown that MHC molecules without any other cell surface protein were sufficient for presentation of processed peptide antigens. This was shown in two ways. First, Watts et al. (184) showed that glass slides coated with lipid containing purified I-A molecules could present an ovalbumin peptide to an ovalbumin specific T cell hybridoma. This result meant that no other special steps were required other than antigen processing and MHC association. Likewise, Walden et al. (185) specifically stimulated T cell hybridomas with liposomes containing nothing but antigen and MHC molecules. Second, Norcross et al. (279) transformed mouse L cells with the genes for the I-A α and β chains and converted the fibroblasts (which do not express their own class II molecules) into I-A-expressing cells. These cells were able to present several antigens to I-A-restricted T cell clones and hybridomas (279), and similar I-E transfectants presented to I-E-restricted T cells (158). Thus whatever processing enzymes are required are already present in fibroblasts, and the only additional requirement for antigen-presenting function is the expression of I-A or I-E antigens.

Biochemical evidence for the direct association between processed peptide and MHC molecules was based first on competition between peptides for antigen presentation (171,280–283) and then more directly on equilibrium dialysis (260), molecular sieve chromatography (261), or affinity labeling (284). Equilibrium dialysis was

performed by incubating detergent-solubilized class II molecules with fluoresceinated or radioactive antigenic peptides, followed by dialysis against a large volume of buffer. In the absence of binding by class II molecules, the labeled peptide would distribute itself equally between the inside and outside of the dialysis chamber. However, when the appropriate class II molecules were added to the chamber, extra peptide molecules were retained inside it due to formation of a complex too large to diffuse across the semipermeable membrane. In this way, direct binding of antigen and MHC was shown, and an affinity constant was determined (260,261).

A second approach was to form the antigen–MHC complex over 48 hr, followed by rapid passage over a Sephadex G50-sizing column. The bound peptide was excluded from the column, since it is the size of class II molecules (about 58 kD), while free peptide was usually included in the column, since it is only approximately 2 kD (Fig. 13) (261). Competitive binding showed that different peptide antigens with the same MHC restriction bind to the same site on the MHC class II molecule (285,286). For example, Table 8 shows the results with peptide antigens that are known to be presented with I-A or I-E antigens of the *d* or *k* haplotype. We observe that Ova peptide 323 to 339, which is presented with I-A^d, also binds well to purified I-A^d, while nonradioactive peptide competes for the antigen binding sites of the I-A^d molecule. Similarly, the other I-A^d-restricted peptide, Myo 106 to 118, competes with Ova 323 to 339 for the same site. However, Myo 132 to 153, which is not restricted to I-A^d, does not compete for it but does compete for its own restriction element, I-E^d. Similarly, pigeon cytochrome *c* competes best for its restriction element I-E^k rather than I-A^k or I-E^d, which do not present cytochrome. Conversely, recombinant E_β genes have been used to map separate sites on a class II MHC molecule for binding to peptide antigen and to the T cell receptor (287).

Using these two biochemical methods, it has been possible to explain major losses of peptide antigenicity resulting from amino acid substitutions in terms of their adverse effect on epitope or agretope function. For example, the response of each of two ovalbumin specific T cell clones was mapped to peptide 325 to 335 by using a nested set of synthetic peptides. Five substitutions were made for each amino acid in this segment, and the resulting 55 different peptides were each tested for the ability to stimulate the clone (241). Presumably, those peptides that failed to stimulate could be defective at an epitope or an agretope functional site. In fact, only two amino acids (Val 327 and Ala 332) were essential for MHC interaction, and changes at either of these resulted in a loss of antigenicity for the clone. Seven other amino acids were critical for T cell stimulation but did not affect MHC binding. Thus these must be part of the functional epitope. Interestingly, certain substitutions for His

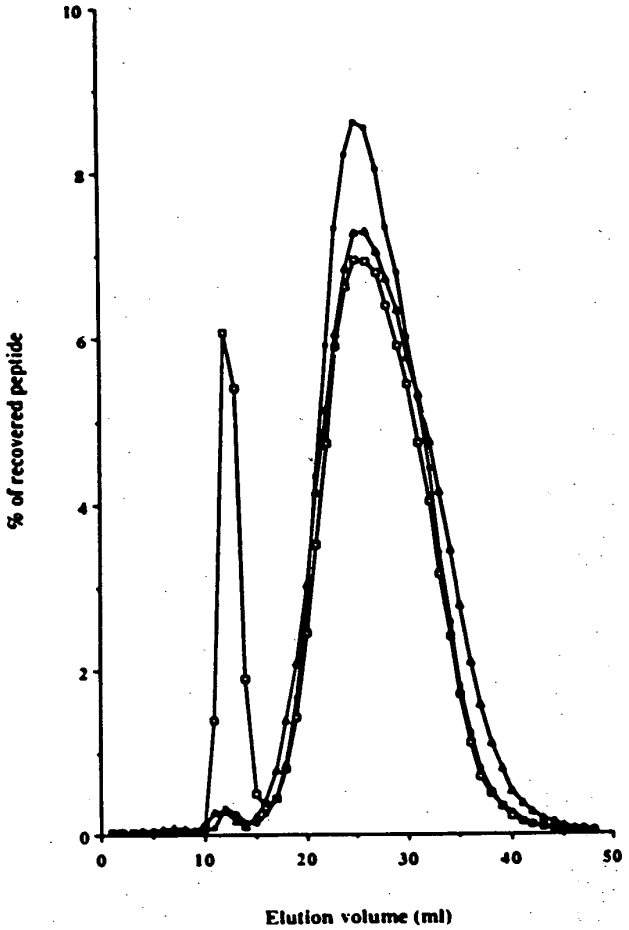


FIG. 13. Gel filtration of OVA 323–339/Ia complexes. Purified I-A^d and I-E^d (or gelatin) in detergent solution was incubated for 2 days at room temperature with [^{125}I]OVA 323 to 339 and subsequently passed over a Sephadex G50 column. The elution volume was collected in 1-ml fractions and counted for radioactivity. (From ref. 261, with permission.)

328, Ala 330, and Glu 333 had effects on MHC binding, while others had effects on T cell stimulation without affecting MHC binding. These amino acids might participate in both agretope and epitope functional sites, or, alternatively, the substitutions may affect the conformation of the peptide as it binds, thus indirectly affecting T cell recognition (240) (see below). The fact that substitutions at 9 of 11 amino acids could be tolerated without affecting MHC binding is consistent with the determinant selection hypothesis in that multiple antigenic peptides are capable of interacting with the same antigen binding site on the MHC molecule.

Similarly, by using a T cell clone specific for peptide 52 to 61 of hen egg lysozyme, substitutions at each amino acid were analyzed for the ability to bind to I-A^k

TABLE 8. *Correlation between MHC restriction and binding to MHC molecules*

Competitor peptide		Ova + A^d	Myo + E^d	HEL + A^k	Cyto + E^k
Ova	323–339	++++	−	++	+
Myo	106–118	++++	−	++	+/−
Myo	132–153	−	++++	−	++
HEL	46–61	+	+	++++	+
Cytochrome *c*	88–104	++	+/−	++	++++

Based on data in ref. 286.

and stimulate the clone (237). Four of 11 amino acid residues were silent, while substitutions at three positions resulted in reduced binding to I-A^k. Substitutions at the remaining three positions resulted in decreased T cell stimulation without affecting MHC association. These sites were organized along the lines predicted by the α-helix model described previously, even though the peptide is not helical when part of the native protein. However, when the peptide is folded as an α helix, the residues of the T cell epitope are lined up on one side and those of the agretope on the other, although the resulting helix is not strictly amphipathic. The epitope was very sensitive to substitutions, even conservative ones such as changing Leu 56 to Ile, norLeu, or Val. The results in both of these studies confirmed by competitive binding that the MHC molecule contains a single saturable site for peptide binding. This site must be capable of binding a broad range of antigenic peptides. It may be through binding the MHC molecule that antigenic peptides become oriented and folded into an ordered structure that is recognizable by the T cell receptor.

Interestingly, one of the first antigenic structures for T cells to be studied in this way was not a peptide at all but rather the small molecule tyrosine–azobenzenearsonate (Tyr-ABA) (130). This molecule provides one of the exceptions to the rule that T cells recognize only peptide antigens, not carbohydrates or haptens. Indeed, other haptens such as trinitrophenyl may bind MHC molecules only when attached to a peptide that binds (288). Nevertheless, the Tyr-ABA structure seems to have some features in common with those discussed above for peptides. It was studied with a series of organic analogs in a classic type of structure–function study. The portion of the structure mapped as the epitope recognized by the T cell receptor was identified to be a combination of the arsonate anionic group and the amino acid zwitterion (NH_3^+, COO^-) of the tyrosine moiety. The component mapped as the moiety interacting with the class II MHC molecule was the planar structure consisting of the two aromatic rings of the tyrosine and the benzenearsonate, linked by the azo linkage. Thus this nonpeptide antigenic structure is amphipathic. In this case, the planar aromatic ring structure could interact with some of the aromatic side chains, which have been found to abound within the floor of the peptide-binding groove of the MHC molecule (242–247). Therefore, although a full set of general principles explaining the specificity of antigen presentation and T cell recognition has not yet emerged, it is studies such as these, combined with complementary structural studies characterizing the antigen-interacting portions of MHC molecules (161,242–246,287,289,290) (see Chapters 16–18) and of T cell receptors (291–294) (see Chapter 11) that will ultimately lead to an understanding of these principles.

One observation that came out of this type of structure–function study was that a single peptide can bind to a class II MHC molecule in more than one way, and thus be seen by different T cells in different orientations or conformations (240,295). This conclusion derived from studying a series of peptides with single amino acid substitutions for recognition by two different T cell clones with the same class II molecule, and competition by inactive peptides with the wild-type peptide for binding to the MHC molecule. Several residues appeared paradoxically to reverse their roles reciprocally for binding the T cell receptor versus binding the MHC molecule when assessed with the two different T cell clones (240). The implication is that one cannot define a unique portion of the peptide that always binds to the MHC molecule, independent of the T cell that is responding, but rather this role may be played by different residues when the peptide is seen by different T cells.

The same conclusion can be reached from an entirely different type of study, in which mutations are introduced into the MHC molecule. Mutations in the floor of the peptide-binding groove, which cannot directly interact with the T cell receptor, can differentially affect recognition of a peptide by one clone and not another (296–298). In a particularly thoroughly studied case, it was clear that the quantitative level of peptide binding was not affected by the mutation, but rather the change in the floor of the groove imposed an altered conformation on the peptide that differentially affected recognition by different T cells (298). If indeed the T cell receptor cannot detect the mutation in the MHC molecule except indirectly by its effect on the peptide conformation, then one is forced to conclude that different T cells have preferences for different conformations of the same peptide bound to (what appears to the T cell as) the same MHC molecule.

Another general observation to come from this type of study is that substitution of amino acids often affects

presentation by MHC and recognition by T cells through introduction of dominant negative interactions or interfering groups, whereas only a few residues are actually essential for peptide binding (299). Both for class II binding (239,299–301) and for class I MHC binding (302), most residues can be replaced with Ala or sometimes Pro without losing MHC binding, as long as a few critical residues are retained. Of course, T cell recognition may require retention of other residues. If many of the amino acid side chains are not necessary for binding to the MHC molecule, then one might expect side chains of noncritical amino acids to have a profound effect in certain cases only by interfering with binding, either directly or through an effect on conformation. That is exactly what was observed when a heteroclitic peptide—that is, one that stimulated the T cells at much lower concentrations than required of the wild-type peptide—was obtained by replacing a negatively charged Glu with Ala or with Gln, which has the same size but no charge (299). An Asp, negatively charged but smaller, behaved like the Glu. Thus this residue was not necessary for binding to the class II MHC molecule, but a negatively charged side chain interfered with binding. These results have important implications for the chemistry of peptide–MHC interaction and for the design of more potent synthetic vaccines.

In the case of class I MHC molecules, recent results defining sequence binding motifs generalize the conclusion that only a few critical "anchor" residues determine the specificity of binding to the MHC molecule (Table 9). These motifs were defined by a detailed study of one peptide–MHC system (303), by sequencing of mixtures of natural peptides eluted from a class I MHC molecule and finding that at certain positions in the sequence a single residue could be found shared by most of the peptides (304), and by separating and sequencing individual natural peptides eluted from a class I molecule and finding a conserved residue at certain positions (255). The latter two studies also made the important observation that the natural peptides eluted from class I MHC molecules were all about the same length, 8 or 9 residues, and this was confirmed for a much larger collection of peptides eluted from HLA-A2 and analyzed by tandem mass spectrometry (305). This finding was consistent with other studies demonstrating that a minimal nonapeptide was many orders of magnitude more potent than longer peptides in presentation by class I molecules to T cells (306,307). This conservation of length was critical to the success of the approach of sequencing mixtures of peptides eluted from a class I molecule (304), because such a method requires that the conserved anchor residues all be at the same distance from the N terminus. Sequencing by Edman degradation is done sequentially from the amino terminus. In a mixture of many unrelated peptides, at most positions one would expect to find close to 20 different amino acids, with no single one standing out, and that is exactly what was found at most positions in the mixture. The fact that Falk et al. (304) could find a single amino acid at certain positions, however, such as a Tyr at position 2 in peptides eluted from K^d, implies not only that most or all of the peptides bound to K^d had a Tyr that could be aligned, but also that the peptides were already aligned as bound to the MHC molecule, with each one having just one residue N terminal to the Tyr. This result implies that the position of the N-terminal residue is fixed in the MHC molecule, even if its side chain can vary, and suggests that the processed peptides must be trimmed to size, so that nothing hangs out of the MHC groove, at least at the N terminus. It is this fact that has made the identification of motifs for binding to class I molecules much more straightforward than finding motifs for binding class II molecules.

This conclusion has not only been confirmed but also explained by the recent x-ray crystallographic data on class I peptide–MHC complexes (245–247). It appears that both the N-terminal α amino group and the C-terminal carboxyl group are fixed in pockets at either end of the MHC groove, independent of what amino acids are occupying those positions, and that the rest of the peptide spans these fixed points in a more or less extended conformation. The minimum length that can span the distance between these pockets is 8 residues, but 9 or 10 residues can be accommodated with a slight bulge or β turn in the middle of the peptide, explaining the narrow restriction on length. Between these ends, one or two pockets in the groove can accommodate the side chain of an amino acid, usually either at position 2 binding in the B pocket, or at position 5 binding in the C

TABLE 9. *Motifs for peptides binding to class I MHC molecules*

Class I MHC molecule	Residue number								
	1	2	3	4	5	6	7	8	9
H-2K^d		Y							I, L, V
H-2D^b					N				M, I
H-2K^b					F, Y			L, M	
HLA-A2.1		L, M							V
HLA-B27	K, R G	R	I, Y, F, W						K, R

Based on refs. 255, 303, and 304.

pocket, depending on the particular MHC molecule. An additional side chain of the C-terminal residue can be accommodated in a pocket at the end of the groove. These residues that fit into pockets correspond exactly to the "anchor" residues, at positions 2 or 5, and 8 or 9, defined by the sequence motifs, and appear to be the primary determinants of specificity for peptide binding, since the rest of the interactions are largely with peptide backbone atoms, including the α amino and carboxyl groups, and therefore do not contribute to sequence specificity. This finding can explain both the breadth of peptides that can bind to a single MHC molecule, because most of the binding involves only backbone atoms common to all peptides, and also the exquisite specificity of binding is determined by the anchor residues that account for the *Ir* gene control of responsiveness.

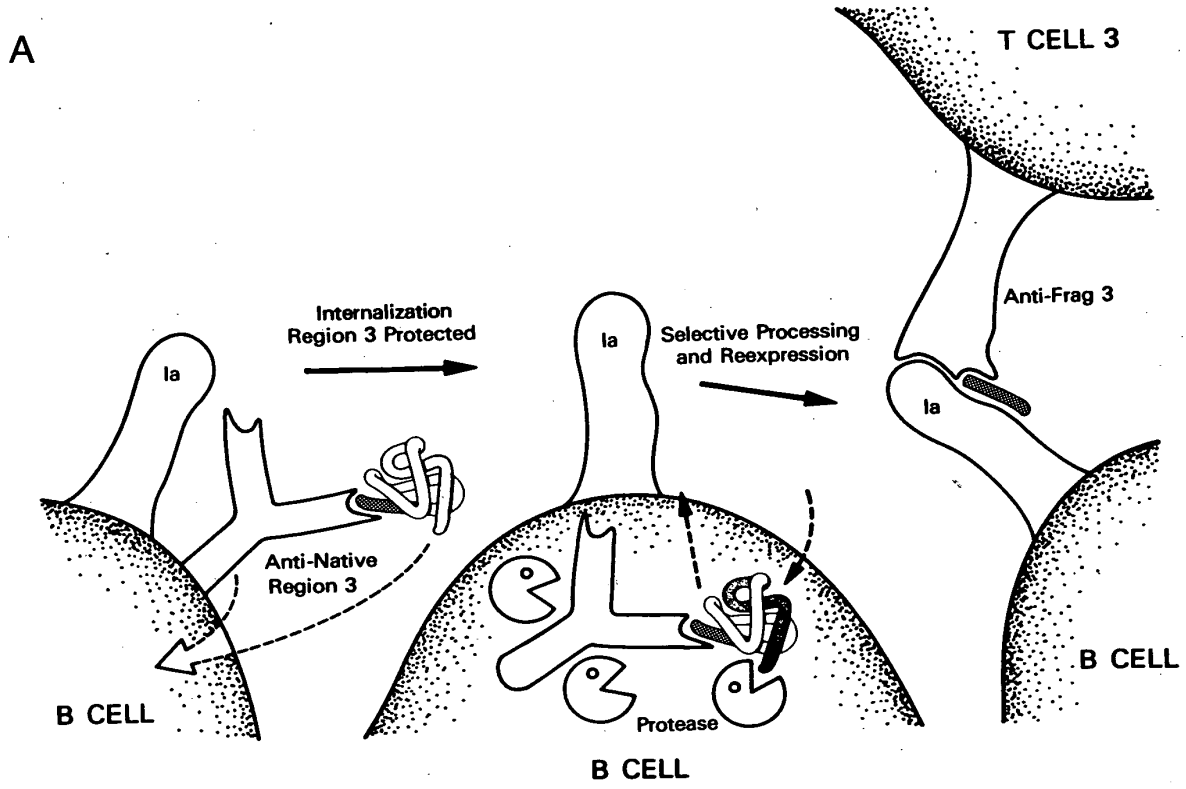
In contrast, when natural self-peptides were eluted from class II MHC molecules (248,308), the lengths were much more variable, ranging from 13 to 18 residues, and several variants of the same peptide were found with different lengths of extra sequence at one end or the other ("ragged ends"). This finding suggested that both ends of the peptide-binding groove of class II MHC molecules are open, in contrast to class I, so that additional lengths of peptide can hang out either side, and trimming does not have to be precise. However, a corollary is that the peptides eluted from class II molecules would not be expected to be naturally aligned in a motif, and that was indeed what was found. Although a moderately conserved motif was found in some of the peptides eluted from the murine class II molecule I-A^d, consistent with the motif defined earlier based on known antigenic peptides binding to I-A^d (309), the motif was neither so clearly defined nor so highly conserved as in the class I case, and required human manipulation to align the sequences to identify a motif (248).

RELATIONSHIP BETWEEN HELPER T CELL EPITOPES AND B CELL EPITOPES ON A COMPLEX PROTEIN ANTIGEN

We have seen that the factors that determine the location of antigenic sites for T cells and for B cells, with the possible exception of self-tolerance, are largely different. Indeed, if B cells (with their surface antibody) bind sites that tend to be especially exposed or protruding—sites that are also more accessible and susceptible to proteolytic enzymes involved in processing—then there is reason to think that T cells may have a lower probability of being able to recognize these same sites, which may be more likely to be destroyed during processing. Certainly, assembled topographic sites are going to be destroyed during processing. On the other hand, there are examples in which T cells and antibodies seem to see the same, or very closely overlapping, sites on a protein (34,143, 158,310–312), although fine specificity analysis usually indicates that the antibody and T cell fine specificities are not identical. The question dealt with here is whether, besides such structural features that T and B cell epitopes may or may not have in common, there are any functional or regulatory factors in T cell–B cell cooperation that would produce a relationship between helper T cell specificity and B cell specificity in the response to a given protein antigen.

The first evidence that helper T cells might influence the specificity of the antibodies produced came from studies of *Ir* gene control of antibody specificity. A number of studies showed that *Ir* genes, which appeared to act through effects of T cell help, could influence the specificity of antibodies produced to a given antigen (139,313–320). An apparent exception was the case of insulin in the guinea pig (321). It is easy to envision how *Ir* genes, which encode MHC molecules that present antigen to T cells, can influence which epitopes of a given protein can be presented to T cells by presenting cells of a given MHC type or how MHC products could influence the T cell repertoire of a given individual. But it is harder to imagine how *Ir* genes could determine which epitopes of a protein elicit antibodies, when such antibodies are generally not MHC restricted. One explanation suggested was that the *Ir* genes first select which helper T cells are activated, and these in turn influence which B cells, specific for particular epitopes, could be activated (102). Because, for cognate help, the B cell has to present the antigen in association with an MHC molecule to the helper T cell, the *Ir* gene control of antibody specificity must operate at least partly at this step by selecting which helper T cell can be activated by and help a given B cell. An example is the case of F_1 hybrid T cells helping parental B cells of one or the other haplotype. Conversely, if the helper T cell selects a subset of B cells to be activated on the basis of their antibody specificity, then there is a reciprocal interaction between T and B cells influencing each other's specificity. Therefore this hypothesis was called "T–B reciprocity" (102). Steric constraints on the epitopes that could be used by helper T cells to help a B cell specific for another particular epitope of the same protein were also proposed by Sercarz et al. (322).

The first attempts to test these ideas involved limiting the fine specificity of helper T cells to one or a few epitopes and then determining the effect on the specificities of antibodies produced in response to the whole molecule. Cecka et al. (323) accomplished this by inducing T cell tolerance to certain epitopes of lysozyme by tolerizing rabbits to cross-reacting lysozymes. Others (100, 324,325) used T cells from animals immune to peptide fragments of the protein. In each case, the limitation on the helper T cell specificity repertoire influenced the repertoire of antibodies produced. An apparent exception was the case of T cells and B cells specific for a decapeptide epitope of tobacco mosaic virus protein, although in


A
T CELL 3
Anti-Frag 3
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Region 3 Protected
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and Reexpression
Ia
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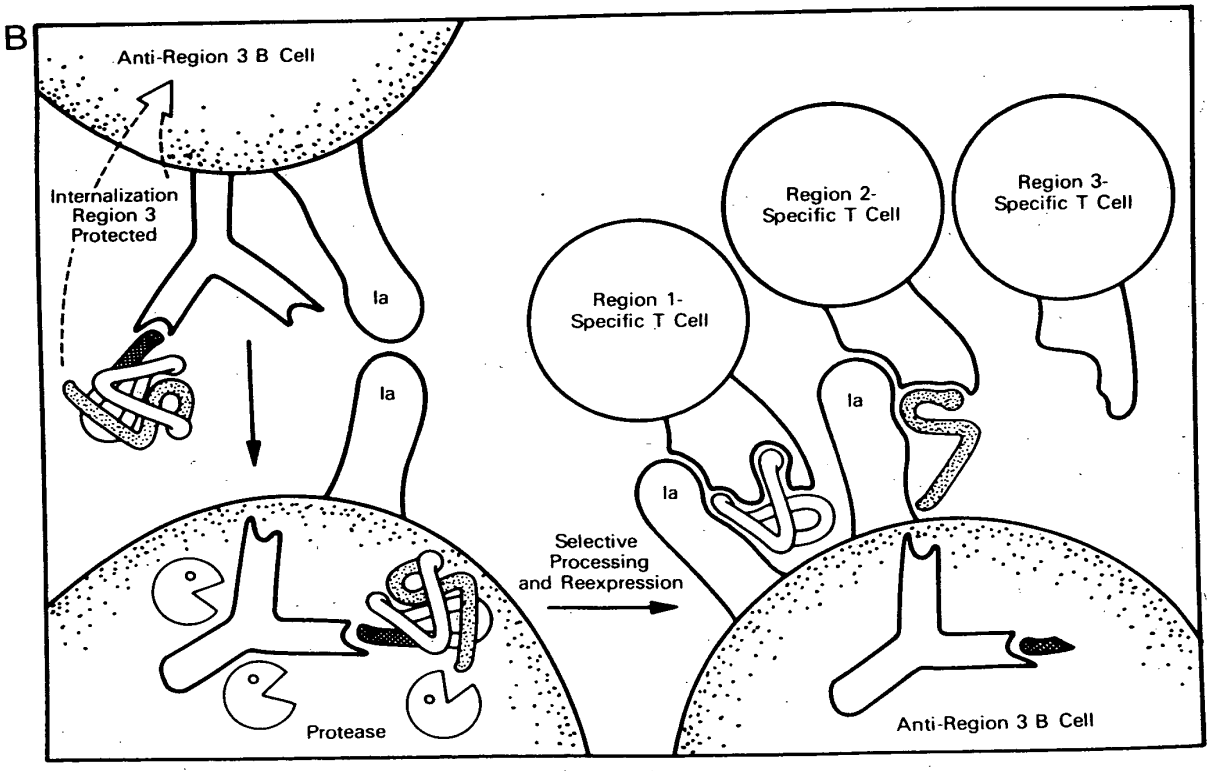
B
Anti-Region 3 B Cell
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Specific T Cell
Region 3-
Specific T Cell
Region 1-
Specific T Cell
Selective
Processing
and Reexpression
Protease
Anti-Region 3 B Cell

this case the selection was largely among T cells and B cells with different fine specificities for the same epitope rather than for different epitopes (326).

At a time when antigen was thought to serve as a direct bridge between the B cell receptor (immunoglobulin) and the T cell receptor, one explanation suggested for these constraints was steric hindrance (102,322). For the antibody, T cell receptor, and MHC to bind simultaneously to the intact protein, the sites they bound in relation to each other were subject to steric constraints. However, it has become apparent that T cells recognize antigen on B cells as on other APCs, not in its intact form but after processing and association with a MHC molecule (327–329). Indeed, these and other studies (330–334) indicated that one purpose of the B cell surface immunoglobulin was to take up the specific antigen with high affinity, but that the antigen was then internalized by receptor-mediated endocytosis and processed like any other antigen. Therefore another explanation was proposed: the surface immunoglobulin, which acts as the receptor to mediate endocytosis, sterically influences the rate at which different parts of the antigen are processed, because what the B cell is processing is not free antigen but a monoclonal antibody–antigen immune complex (102) (Fig. 14). This concept presupposes that many antibody–antigen complexes are stable near pH 6 in the endosome and that what matters is the kinetics of production of large fragments, rather than the products of complete digestion, when both the antigen and the antibody may be degraded to single amino acids. Such protection from proteolysis of antigen epitopes by bound antibody can be demonstrated at least *in vitro* (49). More recently, the effect of antigen specific B cell surface immunoglobulin on the fragments produced by proteolytic processing of antigen was elegantly demonstrated by Davidson and Watts (335). They demonstrated that the pattern of fragmentation of tetanus toxoid, as measured by SDS–polyacrylamide gel electrophoresis, produced during processing by B lymphoblastoid clones specific for tetanus toxoid, varied among B cell clones depending on their specificity for different epitopes within the antigen. Binding to the antibody may also influence which fragments are shuttled to the surface and which are shunted into true lysosomes for total degradation. Thus different B cells bearing different surface immunoglobulin would preferentially process the antigen differently to put more of some potential fragments than others on their surface, in contrast to nonspecific presenting cells that would process the antigen indifferently. By this mechanism, it is proposed that B cell specificity leads to selective antigen presentation to helper T cells and therefore to selective help from T cells specific for some epitopes more than from T cells specific for others (102).

To test this hypothesis from the B cell point of view, Ozaki and Berzofsky (101) made populations of B cells effectively monoclonal for purposes of antigen presentation (but not secretion) by coating polyclonal B cells with a conjugate of monoclonal anti-myoglobin coupled to anti-IgM antibodies. They found that B cells coated with one such conjugate presented myoglobin less well to one myoglobin specific T cell clone than to others. B cells coated with other conjugates presented myoglobin to this clone equally well as to other clones. Therefore the limitation on myoglobin presentation by this B cell population to this particular T cell clone depended on the specificity of both the monoclonal antibody coating the B cell and the receptor of the T cell clone. It happened in this case that both the monoclonal antibody and the T cell clone were specific for the same or closely overlapping epitopes. Therefore it appears that the site bound by the B cell surface immunoglobulin is less well presented to T cells. This finding is also consistent with a recent study of chimeric proteins in which one or more copies of an ovalbumin helper-T-cell determinant were inserted in different positions (336). Although the position of the ovalbumin determinants did not affect the antibody response to an amino terminal site from the heat-stable enterotoxin from *Escherichia coli,* the position did matter for antibody production to the carboxyl terminal determinant of the chimeric protein derived from insulin-like growth factor I. An ovalbumin determinant inserted distal to the carboxyl terminal antibody epitope was much more effective in providing help than one inserted adjacent to the same epitope, when both constructs were used as immunogens, even though both constructs elicited similar levels of ovalbumin specific T cell proliferation in the presence of nonspecific presenting cells *in vitro,* as a control for nonspecific effects of flanking residues on processing and presentation of the helper-T-cell determinants. However, circumstantial evidence from the *Ir* gene studies mentioned previously suggests that T cells may preferentially help B cells that

FIG. 14. Antigen presentation by B cells: positive (**A**) or negative (**B**) selection. A B cell specific for region 3 of a particular antigen internalizes the antigen molecule with the region 3 protected by an antibody against the region. The part of the antigen under the influence of the antibody is protected from proteolytic enzymes (selective processing). In positive selection (**A**), that part is preferentially reexpressed and presented. In negative selection (**B**), the reexpression of that part takes much longer or is much more limited than that for the other parts of the antigen, which are free from the influence of the antibody. Therefore such a B cell can present any part of the antigen, except the one bound to the antibody (region 3), to the relevant helper T cells (selective presentation). (Part **A** from ref. 102, and part **B** from ref. 101, with permission.)

bind with some degree of proximity to the T cell epitope, as there was a correlation between T cell and antibody specificity for large fragments of protein antigens under *Ir* gene control (99,102,139,316,317,320). Therefore antibodies may have both positive and negative selective effects on processing. Further studies on presentation of β-galactosidase–monoclonal antibody complexes by nonspecific APCs suggest similar conclusions (337,338). Presumably, the conjugates are taken up via Fc receptors on the presenting cells and processed differentially according to the site bound by the antibody, so that they are presented differentially to different T cell clones. Thus non-B presenting cells can be made to mimic specific B presenting cells. This also suggests that circulating antibody may have a role in the selection of which T cells are activated in a subsequent exposure to antigen.

Taken together, these results support the concept of T–B reciprocity in which helper T cells and B cells each influence the specificity of the other's expressed repertoire. This mechanism may also provide an explanation for some of the cases in which *Ir* genes have been found to control antibody idiotype (339,340), although certain cases would require another explanation (341). Although we do not yet know all the constraints on the relationships between T and B cell epitopes and therefore cannot yet use this information in a predictive way, these relationships probably play a significant role in regulating the fine specificity of immune response of both arms of the immune system. Therefore they will also be of importance in the design of synthetic or recombinant fragment vaccines that incorporate both T and B cell epitopes to elicit an antibody response.

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The induction of humoral and cell-mediated immune responses to protein antigens requires the recognition of the antigens by helper T cells. The reason for this is that helper T cells are necessary for stimulating B lymphocyte growth and differentiation and for activating the effector cells of cell-mediated immunity, namely macrophages and cytolytic T lymphocytes (CTLs). Before we discuss the activation of T cells by protein antigens and the functions of various T cell subsets in subsequent chapters, we need to understand the structures of antigens that are recognized by T cells. The concept that major histocompatibility complex (MHC)-encoded molecules play an important role in antigen recognition by helper cells and CTLs was introduced in Chapter 5. This chapter describes the formation of complexes of foreign antigens and MHC molecules, which are the ligands for which T cell antigen receptors are specific, and the physiologic significance of this unusual specificity.

CHARACTERISTICS OF ANTIGEN RECOGNITION BY T LYMPHOCYTES

It is now known that $CD4^+$ T lymphocytes, most of which are helper cells, recognize peptides that are bound to class II MHC molecules on the surfaces of other, non-T cells. Class II-associated peptides are usually derived from extracellular microbes and soluble protein antigens. Furthermore, $CD8^+$ T cells, most of which are CTLs, recognize peptide fragments bound to class I MHC molecules on cells that are targets of the lytic action of CTLs. These class I-associated peptides are generally derived from endogenously synthesized proteins, such as viral antigens. The elucidation of these principles was one of the most impressive achievements in immunology in the 1980s. Our current understanding of T cell antigen recognition is the culmination of a vast amount of work beginning with studies on the physicochemical forms of antigens that stimulated cell-mediated immunity. These studies led to the discovery that cells other than T lymphocytes play an obligatory role in T cell activation by foreign antigens and later to the elucidation of the function of MHC molecules in T cell antigen recognition.

Physicochemical Forms of Antigens Recognized by T Lymphocytes

The realization that humoral and cell-mediated immunity are mediated by different classes of lymphocytes, i.e., B and T cells, respectively, led many investigators to examine the properties of the antigens that stimulated these two types of immune responses. Such studies established a fundamental concept, namely that *T lymphocytes recognize different forms of antigens from B lymphocytes and secreted immunoglobulin* (Ig).

1. T lymphocytes recognize only protein antigens, whereas B cells can specifically recognize proteins, nucleic acids, polysaccharides, lipids, and small chemicals. Some T cells are specific for chemically reactive forms of haptens such as dinitrophenol. In these situations, it is likely that the haptens bind to cell surface proteins, including MHC molecules, and these hapten-protein conjugates are recognized by T cells. As we shall see later, the reason why T cells respond only to protein antigens is that only fragments of proteins can form stable complexes with MHC molecules.

2. B cells specific for protein antigens may recognize conformational determinants that exist when proteins are in their native tertiary (folded) configuration or determinants that are exposed by denaturation or proteolysis. In contrast, T cells recognize only linear determinants defined predominantly by primary amino acid sequences. Thus, when an animal is immunized with a native protein, the antibodies it produces will react only with the native protein. In contrast, the antigen-specific T cells which are stimulated by immunization with the native protein will respond to denatured or even proteolytically digested forms of that protein (Table 6-1). Consistent with this difference in the nature of antigenic determinants for T and

TABLE 6-1. Qualitative Differences in Antigen Recognition by T and B Lymphocytes

		Secondary Immune Response	
Immunizing Antigen	**Secondary Antigen Exposure**	***B Cell-Mediated (Antibody Production)***	***T Cell-Mediated (Delayed Type) Hypersensitivity***
Native protein	Native protein	+	+
Denatured protein	Native protein	−	+
Native protein	Denatured protein	−	+
Denatured protein	Denatured protein	+	+

Antigen recognition by T and B lymphocytes is qualitatively different. In an immunized animal, B cells are specific for conformational determinants of the immunogen and, therefore, distinguish between native and denatured protein antigens. T cells, however, do not distinguish between native and denatured protein antigens because T cells recognize non-conformational linear epitopes.

B cell recognition is the finding that T cell responses to a soluble antigen cannot be inhibited using antibodies specific for conformational determinants of that antigen, whereas antigen recognition by B cells can be competitively inhibited by such antibodies.

Role of Accessory Cells in T Cell Responses to Antigens

The second important characteristic of antigen recognition by T lymphocytes is that *T cells recognize and respond to foreign protein antigens only when the antigen is attached to the surfaces of other cells,* whereas B cells and secreted antibodies bind soluble antigens in the circulation or in the aqueous phase. Thus, CTLs recognize antigens bound to the surface of target cells and kill these targets. The activation of helper T cells by foreign antigens requires the participation of cells other than T lymphocytes; these are called **accessory cells.** These accessory cells serve two principal functions in helper T cell stimulation:

1. Accessory cells display fragments of foreign protein antigens on their surfaces in a form that can be specifically recognized by T cell antigen receptors. This phenomenon is called **antigen presentation,** and the cell populations capable of performing this function are **antigen-presenting cells** (APCs). (The term APCs is used for accessory cells that present antigens to helper T lymphocytes. Since CTLs also recognize foreign antigens bound to the surfaces of their target cells, all such target cells may be conceptually included among APCs. Conventionally, however, cells that are recognized and lysed by CTLs are called **target cells,** not APCs.)
2. Accessory cells provide stimuli to the T cell, beyond those initiated by ligand binding to the T cell antigen receptor, which are required for physiologic activation. These stimuli, referred to as **costimulator activities,** are incompletely characterized. They may be provided by membrane-bound or secreted products of accessory cells.

The antigen-presenting functions of accessory cells are described in more detail later in this chapter, and their costimulator functions are discussed in Chapter 7.

The importance of APCs in initiating T cell–dependent immune responses was first suggested in the 1950s by the demonstration that radioactively or fluorescently labeled antigens injected into animals were found in mononuclear phagocytes or follicular dendritic cells and not in lymphocytes. Later studies showed that an antigen that was bound to macrophages *in vitro* and then injected into mice was up to 1000 times more immunogenic on a molar basis than the same antigen administered by itself, in a cell-free form. The explanation for this finding is that T cells respond only to antigen associated with macrophages or other APCs, and only a small fraction of an injected soluble antigen ends up in this immunogenic cell-associated form.

The obligatory role of accessory cells in lymphocyte activation was formally established when techniques for stimulating immune responses *in vitro* were developed. For example, T cells isolated from the blood, spleen, or lymph nodes of individuals immunized with a protein antigen can be restimulated in tissue culture by that antigen. Stimulation may be measured by assaying the production of cytokines by the T cells or by the proliferation of the T cells. When contaminating macrophages and dendritic cells are removed from the cultures, the purified T lymphocytes no longer respond to antigen, and responsiveness can be restored by adding back the macrophages or dendritic cells. Such experimental approaches provide the basis for defining the accessory functions of various cell types in T lymphocyte activation. The importance of accessory cells in immune responses *in vivo* is suggested by the observation that **adjuvants** often need to be administered in addition to antigen in order to elicit an immune response to the antigen. These adjuvants are usually insoluble or undegradable substances that promote nonspecific inflammation, with recruitment of mononuclear phagocytes at the site of immunization.

The Phenomenon of MHC–Restricted Antigen Recognition by T Lymphocytes

The critical advance in our understanding of antigen recognition by helper T cells and CTLs was the discovery of the phenomenon of **self MHC restriction** in the 1970s. *MHC restriction is the requirement that an APC must express MHC molecules that the T cell recognizes as self in order for the T cell to recognize and respond to a foreign protein antigen presented by that APC.* The MHC molecules that T cells recognize as self are those that the T cells encountered during their maturation from precursors in the thymus. (The process of T cell maturation is discussed in much more detail in Chapter 8.) "Self MHC" refers not to MHC molecules expressed by the T cells themselves but to MHC molecules on the APCs or target cells. Normally, because T cells and APCs develop in the same individual, they are syngeneic and all the MHC molecules on the APCs are seen as self MHC by all the T cells in that individual. In experimental systems, T cells respond to antigens presented by a particular APC if the two cell types are at least partly syngeneic, i.e., if they are derived from individuals or inbred strains that share one or more MHC alleles. In this situation the APCs express MHC molecules that the T cells encountered and learned to see as self during their maturation.

This phenomenon of self MHC restriction was discovered when T cells from one inbred strain of animal were mixed with APCs from different inbred strains and T cell responses were assayed. Three sets of ex-